ntramural Activities Volume 1

ctober 1, 1988-September 30, 1989

annual report

Division Of

Cancer Treatment

U S DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

NATIONAL CANCER INSTITUTE

Intramural Activities **Volume 1**

October 1, 1988- September 31, 1989

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Cancer Treatment

U.S. DEPARTMENT OF HEALTH AND
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NATIONAL CANCER INSTITUTE U.S.

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ANNUAL REPORT

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CONTENTS FOR VOLUME I

ANNUAL REPORT

DIVISION OF CANCER TREATMENT

October 1, 1988 through September 30, 1989

The Division of Cancer Treatment (DCT) is the organizational component of the National Cancer Institute (NCI) that is responsible for the identification, development and evaluation of new therapies aimed at the control and cure of cancer. The Division has five major components: Developmental Therapeutics Program (DTP), Cancer Therapy Evaluation Program (CTEP), Radiation Research Program (RRP), Clinical Oncology Program (COP), and the Biologic Response Modifiers Program (BRMP). The research is conducted in intramural laboratories and clinics as well as through grant-, contract-, and cooperative agreement-supported projects throughout the United States and the world. The major emphasis in research efforts of the DCT encompasses chemotherapy, surgery, radiation therapy, immunotherapy, biologic response modifiers, and hyperthermia, used individually and in combination. The conduct of this research follows a stepwise progression and begins in the evaluation of antitumor activity in preclinical tumor systems. Once identified as possessing antitumor activity, the next step is to test and evaluate the safety of the new agent or method of treatment in animals. If the new agent or treatment has an acceptable therapeutic index (i.e., margin of safety between antitumor dose and toxic dose), an investigational new drug (IND) application is submitted to the Food and Drug Administration (FDA). This allows human trials to begin and proceed from Phase I toxicity testing to Phase II antitumor testing and finally to Phase III comparison with existing agents or treatments.

The DCT functions with the scientific advice of the Board of Scientific Counselors (Table I). The Board is composed of leading scientists representing the fields of surgical, medical and radiation oncology, hematology, molecular biology and genetics, immunology, and pharmacology. These represent the areas of clinical and basic science particularly relevant to the research mission of the Division. The Division Director relies on the Board of Scientific Counselors for advice on scientific, administrative and fiscal management of the Division. The Board's counsel ensures that the resources allocated to the DCT are utilized in an optimal fashion.

The scientific accomplishments of the Division over the past year will be presented in detail within this report. In 1989, research achievements ranged from basic observations in the molecular biology of lung cancer to the initiation of human gene transfer experiments in therapeutic trials. The discovery of recessive oncogenes in lung cancer cells introduces many interesting possibilities for future prevention, early detection, and potential therapeutic intervention trials that might introduce critically important suppressor genes. The era of human gene transfer experimentation was initiated this year with the transduction of a gene for neomycin resistance into human tumor-infiltrating lymphocytes. While this highly innovative protocol will utilize this genetic alteration to track the in vivo distribution of these effective cytotoxic lymphocytes, the ultimate objective of this research approach will be used to design cells with enhanced therapeutic effectiveness (e.g., transduction of genes coding for lymphokines into tumor-infiltrating lymphocytes).

Additional highlights of this year included significant progress in the area of drug discovery and development. Following substantial pilot efforts, the antitumor and anti-HIV drug screening program is operational and many exciting new

TABLE I. DCT BOARD OF SCIENTIFIC COUNSELORS

Name	Affiliation	Term of Appointment
John E. Neiderhuber, M.D. (Chairman)	Johns Hopkins University School of Medicine	1986-1990
Robert L. Baehner, M.D.	Children's Hospital, Los Angeles	1988-1992
Charles M. Balch, M.D.	M.D. Anderson Hospital	1987-1991
Yung-Chi Cheng, Ph.D.	Yale University	1986-1990
James D. Cox, M.D.	M.D. Anderson Cancer Center	1987-1991
Lawrence H. Einhorn, M.D.	Indiana University Medical Ctr.	1985-1989
Emil Frei, III, M.D.	Dana-Farber Cancer Institute	1986-1990
Mark T. Groudine, M.D., Ph.D.	Fred Hutchinson Cancer Res. Ctr.	1986-1990
William R. Hendee, Ph.D.	American Medical Association	1987-1990
Susan B. Horwitz, Ph.D.	Albert Einstein College of Med.	1987-1990
William M. Hryniuk, M.D.	Ontario Cancer Foundation	1987-1991
Frank M. Huennekens, Ph.D.	Res. Institute of Scripps Clinic	1988-1991
John Mendelsohn, M.D.	Memorial Sloan-Kettering Cancer Center	1986-1990
Kenneth Olden, Ph.D.	Howard University Cancer Center	1988-1990
Charles E. Putman, M.D.	Duke University Medical Center	1986-1989
Geraldine Schechter, M.D.	VA Medical Center, Washington, DC	1985-1989
Robert T. Schimke, M.D.	Stanford University	1986-1989
H. Rodney Withers, M.D., D.Sc.	UCLA Center for Health Sciences	1986-1989

agents have been identified. The commitment to successful drug development for novel agents is exemplified this year by the studies of new antiretroviral agents. A Phase I investigation of dideoxyinosine has demonstrated improvement in T_4 cells and a decrease in the viral load (HIV p24 antigen) of patients with AIDS. Pediatric studies involving AZT have shown clinical benefit for children with neurodevelopmental deficits secondary to AIDS. Innovative combinations of antiretroviral agents are being piloted in both adult and pediatric patient populations. Finally, encouraging preliminary data indicate that suramin has activity in prostatic carcinoma, and confirmatory trials are under way.

PERSONNEL AND ORGANIZATION

The DCT is operationally divided into five major components of treatment program priorities. Each program is headed by an Associate Director who is responsible for the overall direction of the science within that program. Personnel changes that have occurred during the past year include the following:

A. Office of the Director (OD)

- Dr. Michael R. Grever has been appointed Deputy Director of the Division; he comes to DCT from Ohio State University, where he was Professor of Medicine and Physiological Chemistry and Director of the Interdisciplinary Oncology Unit.

B. Biological Response Modifiers Program (BRMP)

- Dr. Morris Kelsey was recruited as a Health Scientist Administrator in the Biological Resources Branch; he was formerly with the Division of Research Grants, NIH.

- Dr. John Janik was recruited as an Expert in the Clinical Research Branch; he was formerly at the Center for Health Research, Research Triangle Institute.

- Dr. Robert Fenton was recruited as an Expert in the Clinical Research Branch; he was formerly at the Brigham and Women's Hospital of the Harvard Medical School.

- Dr. William Sharfman was recruited as an Expert in the Clinical Research Branch; he was formerly at the Cleveland Clinic.

- Dr. John O'Shea was recruited as an Expert in the Laboratory of Experimental Immunology; he was formerly with the National Institute of Child Health and Human Development, NIH.

- Dr. Bonnie Mathieson, Senior Investigator in the Laboratory of Experimental Immunology, transferred to the National Institute of Allergy and Infectious Diseases, NIH.

- Dr. Howard Young transferred from the Laboratory of Molecular Immunoregulation to the Laboratory of Experimental Immunology as Head, Cellular and Molecular Immunology Section.

- Dr. Sukadev Lavu, Senior Staff Fellow in the Lab. of Biochemical Physiology, transferred to the U.S. Army Medical Res. Institute of Infectious Diseases.

C. Cancer Therapy Evaluation Program (CTEP)

- Dr. Robert Wittes resigned as Associate Director, CTEP, to become Senior Vice President for Cancer Research, Bristol-Myers Co., Wallingford, Connecticut.

- Dr. Michael Friedman, formerly Chief of the Clinical Investigations Branch, was appointed Associate Director, CTEP.

- Ms. Linda Hogan, Head, Protocol and Information Office, CTEP, resigned to assume a management position at ELM Services, Rockville, Maryland.

- Dr. Richard Ungerleider, formerly Head, Pediatric Section, Clinical Investigations Branch, was appointed Chief, Clinical Investigations Branch.

- Dr. Stacy Nerenstone resigned as Medical Officer, Clinical Investigations Branch, to go into private practice in New Haven, Connecticut.

- Dr. Timothy Chen was recruited as a Mathematical Statistician in the Biometric Research Branch; he was formerly a Senior Statistician at Alcon Laboratories in Fort Worth, Texas.

- Dr. Edward Korn was recruited as a Mathematical Statistician in the Biometric Research Branch; he was formerly Associate Professor in the Dept. of Biomathematics, UCLA School of Medicine and Jonsson Comprehensive Cancer Ctr.

- Dr. Walter Torri will spend a year as a Special Volunteer, from Italy, in the Biometric Research Branch.

- Dr. Sylvain Durrleman resigned as an Expert in the Biometric Research Branch to accept a position at Hopital Villejuif, Paris.

- Dr. Michael Hawkins was appointed Chief, Investigational Drug Branch; he had been Head, Biologics Evaluation Section, Investigational Drug Branch.

- Dr. Jason Fisherman was recruited as a Medical Officer in the Investigational Drug Branch; he was formerly Research Fellow at Children's Hospital, Boston.

- Dr. Lorraine Cazenave was appointed a Medical Officer in the Investigational Drug Branch; she was formerly a Medical Staff Fellow in COP, DCT.

- Dr. J. Mel Sorensen was recruited as a Medical Officer in the Investigational Drug Branch; he was formerly Sr. Clinical Research Fellow at the Mayo Clinic.

- Dr. Gisele Sarosy transferred from the Investigational Drug Branch to the Physicians Data Query Section, Office of the Director, NCI.

- Dr. Jean Grem transferred from the Investigational Drug Branch to the Medicine Branch, Clinical Oncology Program, DCT.

- Dr. Brian Leyland-Jones resigned his Expert position in the Investigational Drug Branch to become Professor and Chairman, Department of Oncology, McGill University, Montreal.

- Dr. Michaele Christian was appointed Head, Developmental Chemotherapy Section, Investigational Drug Branch; she was formerly a Medical Officer in that Section.

D. Clinical Oncology Program (COP)

- Dr. Gregory Curt has been appointed Associate Director, COP; he returns to DCT from the Roger Williams Hospital, Brown University, where he was Director of Medical Education and Chief of Clinical Pharmacology.

- Dr. Daniel C. Ihde, Deputy Chief, NCI-Navy Medical Oncology Branch, is now also the new Editor-in-Chief of the JOURNAL OF THE NATIONAL CANCER INSTITUTE.

- Dr. James L. Mulshine has been appointed Head of the newly established Biotherapy Section of the NCI-Navy Medical Oncology Branch.

- Dr. Ilan Kirsch has been appointed Head of the newly established Acquired Gene Rearrangements Section of the NCI-Navy Medical Oncology Branch.

- Sarah Barksdale and Rhoda Alani, members of the Howard Hughes Medical Institute Research Scholars Program, completed their year of research training.

- Dr. Charles E. Myers has been appointed Chief, Medicine Branch.

- Ms. Jean Jenkins, Special Assistant for Clinical Affairs, Medicine Branch, has left NCI to become Head, Cancer Nursing, Clinical Center, NIH.

- Dr. Dwight Kaufman joined the staff of the Medicine Branch as a Senior Investigator; he comes to DCT from Roswell Park Memorial Institute.

- Dr. Leonard Neckers joined the staff of the Medicine Branch as a Research Chemist; he comes to DCT from Laboratory of Pathology, Division of Cancer Biology and Diagnosis, NCI.

- Dr. Emile Y. Brouwers joined the Pediatric Branch as a Visiting Scientist, from the Netherlands; he is functioning as a Clinical Psychologist for the Pediatric AIDS patients.

- Dr. Zelig Tochner has been appointed Cancer Expert in the Radiation Oncology Branch.

- Dr. Thomas Goffman has been appointed Head of the Radiotherapy Section, Radiation Oncology Branch.

- Dr. Judith Bader, a Radiation Oncologist with the Radiation Therapy Section, Radiation Oncology Branch, has left to go into private practice in Silver Spring, Maryland.

- Dr. Jane Grayson, Chief of the Radiation Oncology Branch Navy Section, has left to go into private practice in Alexandria, Virginia.

E. Radiation Research Program (RRP)

- Mr. Robert J. Morton, Jr., retired from the Commissioned Corps as Senior Health Services Officer, Radiotherapy Development Branch, to become Manager of Regulatory Affairs for Siemens Medical Laboratories in California.

- Dr. Robert Holden, Chief of Diagnostic Radiology at the University of Indiana Medical Center, joined the RRP under the Interagency Personnel Act for a one-year sabbatical.

F. Developmental Therapeutics Program (DTP)

- Dr. Saul Schepartz was appointed Cancer Expert in the Office of the Associate Director; he returns to DCT from the University of Medicine and Dentistry of New Jersey.

- Dr. David Clanton was appointed Cancer Expert in the Office of the Associate Director; he transferred to DCT from the Division of Cancer Etiology, NCI.

- Dr. Ralph Collins was recruited as a Cancer Expert in the Natural Products Branch; he was formerly with the University of Connecticut.

- Dr. Daniel Lednicer was recruited as a Cancer Expert in the Drug Synthesis and Chemistry Branch; he was formerly with Adria Laboratories in Columbus, Ohio.

- Mr. J. Paul Davignon, Chief, Pharmaceutical Resources Branch, left to accept the position of Vice President of Manufacturing and Distribution, U.S. Bioscience, Blue Bell, PA.

- Mr. Lawrence Trissel of the Pharmaceutical Resources Branch left to accept the position of Director of Investigational Pharmaceutical Services at M.D. Anderson Cancer Center in Houston.

- Ms. Anastasia Perez joined the Pharmaceutical Resources Branch as a Pharmacist; she was formerly with the Food and Drug Administration.

- Mr. Raymond Greene joined the Pharmaceutical Resources Branch as a Formulation Chemist; he was formerly with the Food and Drug Administration.

- Dr. Kenneth Paull was appointed Chief, Information Technology Branch.

- Dr. Walter Hubbard, Pharmacologist, Program Development Research Group, left to accept a position at Johns Hopkins University.

- Dr. Steven Coval was recruited as a Staff Fellow in the Program Development Research Group; he was formerly with Cornell University.

- Dr. Murray Munro was recruited as a Visiting Scientist in the Program Development Research Group; he was formerly at the University of Canterbury, New Zealand.

- Dr. Peter Roller was appointed Research Chemist, Laboratory of Medicinal Chemistry; he transferred from the Division of Cancer Etiology, NCI.

- Dr. Terrence Burke was recruited as a Research Chemist in the Laboratory of Medicinal Chemistry; he was formerly with Peptide Technologies Corp., Washington, D.C.

PROGRAM REVIEWS

The Division has five scientific programs that are described in detail in their respective individual sections of this Annual Report. Program highlights are listed below:

CLINICAL ONCOLOGY PROGRAM (Associate Director, Gregory Curt)

The Clinical Oncology Program (COP) is the intramural treatment research arm of the National Cancer Institute. The Program conducts basic and clinical research in medicine, surgery, pediatrics, radiotherapy and radiobiology, pharmacology, immunology, genetics and endocrinology in the context of developing curative therapies for cancer. The COP is divided into the Medicine Branch (Chief, Dr. Charles E. Myers), NCI-Navy Medical Oncology Branch (Chief, Dr. John Minna), Surgery Branch (Chief, Dr. Steven A. Rosenberg), Pediatric Branch (Chief, Dr. Philip Pizzo), and Radiation Oncology Branch (Chief, Dr. Eli Glatstein). A laboratory under the

supervision of Dr. Samuel Broder (Director, NCI) operates under the Office of the Associate Director and is directed at identifying new treatments for AIDS. This office also supports a Biostatistical and Data Management Section, headed by Dr. Seth Steinberg.

Medicine Branch

The Medicine Branch has been structurally reorganized to provide disease-specific orientation to clinical research and patient-care delivery. Disease-specific clinics are now manned by protocol principal investigators. The reorganization has improved both educational opportunities and patient management in the clinic setting. The Office of Protocol Management has been established to provide the necessary support for the newly organized Clinical Research Program. This office supervises the entire process of protocol design, approval, and implementation. This office works in close concert with the Biostatistical and Data Management Section under the Office of the Associate Director, COP. The reorganization of the Medicine Branch with the improved systems for data acquisition and management will enhance the ultimate mission of this Branch, which is to interface laboratory and clinical medicine in the process of developing highly innovative treatment modalities for human malignancy.

Several research accomplishments include:

A. Accomplishments in Cancer Research

Suramin has been extensively investigated. Activity in the treatment of metastatic prostatic carcinoma has been observed. Preliminary results suggest that 40-60% of the patients had some evidence of improvement following administration of suramin, including a 50% response rate in patients with measurable disease.

GM-CSF has been explored for its potential to decrease the toxicity of carboplatin in patients with ovarian carcinoma failing to respond to frontline treatment. The initial response rate of 50% is very encouraging in this group of previously treated patients.

Collaborative research demonstrated an association between leukocyte platinum-DNA adduct level and subsequent response to cisplatin in ovarian carcinoma. This observation raises the potential that this technique may enable the clinician to distinguish at an early point the responders from the non-responders.

Through the isolation and characterization of drug-resistant mutants, the regulation of thymidylate synthase activity at the level of message translation has been identified as an important site of tumor cell modulation of response.

Important laboratory advances demonstrated that interferon reverses the overexpression of thymidylate synthase.

10-Formyl-dihydrofolate a metabolite of folate generated in the presence of methotrexate, has been shown to be a potent inhibitor of thymidylate synthase and GAR transformylase.

The sulfones appear to be unusually effective inhibitors of Toxoplasma gondii dihydropteroate synthetase and thus show promise in the treatment of toxoplasmosis in AIDS patients.

The gene for a putative folate transporter in normal and tumor cells has been cloned.

The development of multidrug resistance is associated with the loss of estrogen receptors and marked increase in EGF-receptor expression in breast cancer.

The mechanism of uptake of oligonucleotides by cells along with the basic structural requirements for this process were identified.

B. Accomplishments in Antiretroviral Therapy

The Medicine Branch has had a major commitment to developing effective retroviral therapy for human disease. The technology to detect drugs or biologics that suppress replication of HIV in vitro has been developed. There has been a major emphasis on bringing new anti-AIDS drugs to clinical trials as quickly as possible. Currently, an emphasis has been placed on utilizing the information gained on these new agents to develop an optimal strategy for therapy of AIDS.

Investigators within the Medicine Branch have pioneered AZT and completed Phase I investigations of dideoxycytidine (ddC) and dideoxyinosine (ddI). The most recently completed Phase I trial has demonstrated that ddI increased T4 cells subsets and decreases the viral load. The Medicine Branch has laid the essential groundwork for future Phase II trials of several new agents in the treatment of AIDS.

Combination studies to explore weekly AZT alternating with ddC have been developed to minimize the toxicity of each agent (neuropathy and myelosuppression). Highly innovative studies to combine GM-CSF and AZT have been initiated, and ten patients have been carefully studied. GM-CSF can potentiate the bone marrow function in AZT-treated patients, and the alternating regimen of GM-CSF with AZT is clinically active in suppressing viral replication in vivo. While this combination approach may enhance the tolerance of AZT, in vitro data had been produced that demonstrated that GM-CSF increased AZT entry into macrophages along with increased phosphorylation of the agent. These observations indicate that the combination of GM-CSF and AZT may increase the anti-HIV activity in vitro.

The program continues to strive for the development of both new tools and agents for intervention against this devastating disease process. New drugs are under investigation, including: an epoxy congener of cytidine; newly synthesized compounds including adenalline and cytallene; agents that block viral binding to the cell surface; and inhibitors of viral replication (oligodeoxynucleotides).

In summary, the Medicine Branch has made a major commitment to meeting the challenge of this devastating retroviral disease.

NCI-Navy Medical Oncology Branch

A. Accomplishments in Cancer Research

Extensive resources have been devoted by this Branch to identifying the basic molecular pathobiology of lung cancer. These research activities are critically important not only in enhancing our understanding of carcinogenesis, but provide the potential for innovative therapeutic intervention in the treatment of this extremely common form of cancer. Ongoing studies have identified abnormalities in

the rb gene in potentially all small cell lung cancers and several non-small cell lung cancers. Recent studies have identified p53 as a major recessive oncogene in over 50% of all types of lung cancers. The investigations have potential direct bearing on future prevention and prognostic studies and direct the search toward early molecular detection of lung cancer and/or the detection of patients exhibiting some of these abnormalities in a premalignant phase. Furthermore, studies are ongoing to try to "correct" the malignancy in lung cancer cells by reintroducing the suppressor genes into lung cancer cells through transfection with retroviral vectors.

Dominantly acting oncogenes have also been investigated. Deregulated expression of some oncogene family members (jun) has resulted in loss of control of normal regulatory signals (e.g., serum and phorbol esters). The observation that deregulated expression of normal oncogene members in concert with a mutated ras gene (that can transform normal embryo cells to malignancy) suggests that a relationship exists between these transcription factors and stimulation by tumor promoters. Lung cancer cells may result from a state of chronic tumor promotion. Therefore, vector and transfection techniques are being developed for delivering dominant negative oncogenes into cancer cells as well as the deliverance of tumor suppressor genes. In addition, the use of these techniques to deliver oncogenes into normal bronchial epithelial cells will provide an opportunity to study the process of carcinogenesis on a fundamental level.

Extensive studies of peptide hormones, growth factors, receptors and signal transduction pathways are actively being pursued by this Branch. For example, an insulin-like growth factor has been found to function as an autocrine growth factor for all types of lung cancer and colon cancer, thus providing a potential new target for antitumor therapy and diagnosis. Other potential targets for antitumor therapy in lung cancer include: specific receptors for various peptides that have been found to stimulate the growth of lung cancer cells in vitro; receptors for nicotine (as a potential promoter of lung cancer growth in vitro); atrial naturetic factor; and cholera toxin as a potential inhibitor in vitro of growth of lung cancer cell lines. Mechanisms of drug resistance and sensitivity in both lung and colon cancer are being explored. Monoclonal antibodies developed against lung cancer cells have also been studied for their potential use in early-detection studies. Prospective cooperative group studies to test the validity of these early findings have been designed and implemented.

B. Accomplishments in Antiretroviral Research

A search for retroviral involvement in human T-cell lymphoma and lung cancer is under way. Subsequently, clinical trials are being developed to test drugs known to be effective inhibitors of HIV replication as treatment for human cutaneous T-cell lymphoma. Cytogenetic and molecular biologic tools are being utilized to better characterize various types of lymphoma and leukemia with a particular focus on the T-cell subsets of these basic disease processes.

Pediatric Branch

Investigators within the Pediatric Branch have conducted both basic and clinical investigations on a variety of clinically important areas for both pediatric and adult patients with malignancy. Major research efforts have focused on the CNS involvement with malignancy, pharmacology of various antineoplastic agents,

assessment of dose-intensity in the treatment of various malignancies both with and without GM-CSF administration, significant contributions in the area of antiretroviral therapy for HIV infection in children. In addition, basic science investigations have been conducted to examine the problem of drug resistance in leukemia and lymphoma. The utility of the polymerase chain reaction technique to evaluate minimal residual disease in small non-cleaved lymphomas has been explored.

Several major achievements include the following observations over the past year:

A. Accomplishments in Cancer Research

An extensive longitudinal assessment of neuropsychcological function in children who received cranial radiation and intrathecal chemotherapy in contrast to patients receiving high-dose methotrexate for CNS prophylaxis was conducted. There was no adverse effect of high-dose methotrexate on cognitive function. In contrast, patients receiving combined therapy with radiation therapy and intrathecal chemotherapy did manifest significant impairment of academic achievement. The value of high-dose methotrexate in CNS prevention therapy for children with ALL was confirmed.

Effective CNS prevention therapy can be achieved in high-risk patients with ALL without the use of cranial radiation. The use of aggressive, early intensification chemotherapy and intensive systemic maintenance therapy together with CNS-directed specific therapy was highly effective in high-risk patients. This intensive approach included the systemic administration of high doses of methotrexate and cytosine arabinoside, and intrathecal cytosine arabinoside and methotrexate.

Average-risk patients with ALL can receive effective CNS prophylactic therapy with intrathecal methotrexate alone and do not require high-dose methotrexate.

Intrathecal diaziquone (AZQ) was demonstrated to have definite clinical activity in refractory meningeal malignancy, at a dose that was not associated with clinical toxicity.

Intrathecal mecaptopurine was investigated in a Phase I trial. No significant toxicity has yet been observed, and the results indicate that this agent is safe and active against meningeal leukemia.

Thiotepa administered intravenously is being investigated in conjunction with the administration of GM-CSF. This agent may have utility in the treatment of CNS malignancy when administered by the intravenous route.

Dose-intensity is a critically important element in the successful treatment of small non-cleaved lymphomas. The concomitant administration of colony-stimulating factors may permit more successful therapy by enhancing the ability to administer maximal doses of myelosuppressive chemotherapeutic agents in the treatment of both lymphomas and metastatic sarcomas.

Basic studies have demonstrated that acquired resistance in ALL may involve expression of both mdr-1/P-170 and expression of GST (drug-metabolizing enzymes overexpressed in certain breast cancer cell lines). Furthermore, molecular biologic studies in Burkitt's lymphoma have indicated that molecular abnormalities may provide targets for specific therapeutic endeavors. Antisense oligonucleotide

directed against intron sequences present in the mature messenger RNA species in Burkitt's lymphoma may inhibit the expression of c-myc gene in a subset of patients. Preclinical studies investigating the use of antisense oligonucleotides against Burkitt's lymphoma xenografts in the nude mouse model are under way.

B. Accomplishments in Antiretroviral Therapy

The administration of AZT to the pediatric population has been of definite benefit in children with neurodevelopmental deficits due to the disease. Current studies are investigating the simultaneous use of AZT and GM-CSF and alternating AZT and ddC. A recently initiated study for ddI is also demonstrating encouraging early results.

Radiation Oncology Branch

Investigators within the Radiation Oncology Branch (ROB) continue to have collaborative interaction with other clinical branches in conducting multimodality therapeutic trials. The primary study within the ROB has focused on the treatment of patients with Stage I and II breast cancer. Patients are randomized to receive definitive radiation therapy with breast preservation following lumpectomy or to undergo a modified radical mastectomy. This study differs from the published NSABP program in that the surgical excision in the ROB study makes no attempt to have margins surgically free of tumor, but simply removes the gross tumor at the time of surgical intervention. This trial has accrued 240 patients to date, and long-term follow-up so far has failed to demonstrate superiority of either arm.

Radiation potentiation by the concomitant use of halogenated pyrimidines is being investigated in both sarcomas and gliomas. The investigation of photodynamic therapy has been initiated. In the laboratory, basic research is focused on the mechanisms of sensitization and protection resulting from radiation modifiers and investigation of mechanisms of action of several different cytotoxic agents.

Surgery Branch

Investigators in the Surgery Branch have continued to perform highly innovative clinical trials to define the optimal adoptive immunotherapy. In addition, various combinations of cytokines have been examined clinically with results being correlated with basic investigation. Specific achievements include: the administration of lymphocyte-activated killer cells and interleukin-2 or the administration of high-dose interleukin-2 alone has been confirmed to produce objective responses in patients with metastatic melanoma or metastatic renal cell carcinoma.

A clinical trial has been completed in patients with advanced malignant melanoma demonstrating that tumor-infiltrating lymphocytes plus IL-2 has a 50-60% response rate.

The combination of α-interferon and IL-2 in escalating doses has been demonstrated to have a response rate that is higher than the administration of each cytokine alone.

Clinical investigations have been either completed or initiated with GM-CSF in combination with cytotoxic chemotherapy, IL-2 in combination with monoclonal antibody and LAK cells, escalating doses of IL-4, and photodynamic therapy studies in patients with intraperitoneal malignancies and bronchial obstruction from tumor.

The Surgery Branch has collaborated with the Radiation Oncology Branch in conducting several important studies, including: a prospectively randomized trial of intraoperative radiation therapy versus external-beam radiation therapy for patients with either pancreatic carcinoma or retroperitoneal sarcoma; a prospectively randomized study for patients with either Stage I or II breast carcinoma in comparison to modified radical mastectomy; and a study evaluating intensive preoperative chemotherapy for women with locally advanced stages of breast carcinoma.

Finally, gene transfer studies in vivo have been initiated. Experimental techniques have been developed for inserting foreign genes into tumor-infiltrating lymphocytes. These landmark studies with gene transfer, hopefully, will form the basis for subsequent well-designed studies that may alter identified genetic material as a form of highly innovative anticancer treatment.

RADIATION RESEARCH PROGRAM (Associate Director, John Antoine)

Investigators within the Radiation Research Program (RRP) are responsible for coordinating research activities in this extramural program with two branches: the Diagnostic Imaging Research Branch (DIRB), and the Radiotherapy Development Branch (RDB). The accomplishments of this program will be highlighted:

Neutron-beam therapy continues to be evaluated in localized prostatic cancer, head and neck tumors, and radioresistant neoplasia. A limited number of institutions continue to treat clival chordomas, base-of-the-skull chondrosarcomas, and uveal melanomas with proton-beam therapy.

Intraoperative radiation therapy continues to be investigated in advanced local gynecologic and rectal tumors, retroperitoneal sarcomas, and gastric cancer. Radiation modifiers are currently under clinical investigation. SR-2508, which is a radiosensitizer, is presently being investigated in cooperative-group Phase II and III clinical trials. Furthermore, WR2721, which is a radioprotective agent, is being investigated with chemotherapy in addition to radiation.

Photodynamic therapy remains a high research priority. Utilization of this modality for malignancies such as carcinoma of the ovary, mesothelioma, and bladder cancer is being explored. Efficacy of photodynamic therapy in re-establishing a patent airway in tumors of the lung is also being demonstrated.

Research to determine optimal radiation treatment planning procedure. Symmetry studies, development of systems to rapidly and automatically extract the anatomic features and assist in treatment planning, and studies to examine "patterns of care" are under way.

Several areas of continued research are of importance in diagnostic radiology. The Radiologic Diagnostic Oncology Group (RDOG) has been organized under the leadership of Dr. Barbara McNeil, Professor of Radiology at Brigham and Women's Hospital, Boston. The first prospective diagnostic imaging trials are focusing on prostate and lung cancer. Subsequent studies will embark on a similar program for pancreatic and colorectal cancer. Magnetic resonance imaging and spectroscopy are being utilized to evaluate tumor diagnosis and response.

PET scans have potential utility in evaluating differentiation of normal brain from brain tumors and determination of tumor viability following therapy with radiation therapy. The possibility of using imaging techniques such as MRI and

PET scanning to detect early involvement of the brain with nonmalignant processes (to evaluate central nervous system involvement in the sero-positive asymptomatic AIDS patient) is being considered.

DEVELOPMENTAL THERAPEUTICS PROGRAM (Associate Director, Michael Boyd)

This comprehensive program involves both intra- and extramural scientists who are committed to the preclinical development of the most promising new agents for the treatment of cancer or AIDS. Program accomplishments over the past year include:

A. Accomplishments in Cancer Research

DTP has implemented a novel primary anticancer drug screen that utilizes a broad *in vitro* panel of human tumor cell lines for the discovery of compounds with potential antitumor activity.

The antitumor drug-screening program has been completely reorganized in an effort to increase the yield of identification of successful anticancer agents. The ultimate configuration of the screening panel will encompass a hundred or more cell lines representing 10 to 12 different cancer types. At present, 60 human tumor cell lines were derived from seven cancer types to establish the initial pilot screening panel.

Over 30 of the *in vitro* human tumor panel lines of the disease-oriented drug discovery program have been adapted and characterized for growth as xenografts in athymic nude mice for secondary drug evaluation models; colon and melanoma cell lines have been the most readily established *in vivo*. Other tumor models are undergoing further refinement. The availability of these xenograft lines is an important complement of the present drug evaluation and development strategy.

Extensive work has been completed to evaluate the optimal screening assay. The sulfrohodamine-B assay performed satisfactorily has practical advantages for large-scale screening of anticancer compounds.

A program-wide effort for new drug discovery from natural products continues to undergo development and implementation. This represents an interdisciplinary effort coordinated through the Frederick Cancer Research Facility (FCRF) and involves key participants of both intra- and extramural scientists within DTP.

Intramural program initiatives included extensive work on assay development for primary antitumor screens, analysis of disease-specific selective antitumor drug activity, the development of assays for testing AIDS antiviral compounds (confirmation of antiviral activity, definition of the mechanism of action, and an assessment of cellular and viral range of activity). Comparative testing and ranking of these compounds is also given significant attention.

Extensive work has been done to evaluate potential problems that are associated with the various assays for assessing tumor cell viability. The development of a CO_2-independent culture method for use in the *in vitro* assays will facilitate the anticancer drug screening program.

Cell lines that are derived from human carcinomas are widely used for studying human tumor biology and experimental therapeutics. The validity of these cell

lines as "disease-oriented" is dependent on whether they retain the characteristics of their respective parent cancers. Over 100 commonly used human carcinoma cell lines derived from six types of cancer were analyzed. Extremely careful characterization was accomplished to confirm the tissue of origin, tumor type, degree of differentiation and tumor biology of the cell lines cultivated under both in vitro and in vivo propagation techniques. Approximately 20% of the cell lines were diagnosed as undifferentiated carcinomas and could not be further classified. The majority of the other cell lines were very poorly differentiated but retained sufficient characteristics to allow classification consistent with the other parent cancer. Fewer than 20% of the cell lines were found to retain the spectrum of characteristics generally associated with the parent cancer type. The findings of this study stressed the importance of detailed characteristization of cell lines prior to their utilization as valid models for an in vitro study of human cancer.

Extensive biochemical characterization of lung cancer cell lines and lung tumor tissue from individual lung cancer patients has been accomplished. The ability to propagate human lung tumors in the lungs of nude mice by a novel intrabronchial and intrathoracic implantation method has allowed for improved in vivo propagation, biochemical and molecular genetic as well as drug screening studies. The ultimate goal is to develop a model for pharmacoselective drug treatment based on the biochemical and the molecular genetic profiles of individual lung tumors and corresponding normal lung tissue.

In addition to acquisition of promising anticancer and antiviral compounds, investigators within the DTP have maintained a resource for the production, quality-control and distribution of disease-free experimental animals for research purposes.

An extensive automated information resource has been developed over the past year. This information tracking capability was necessary to enhance the integration of the screening laboratory for computer systems with the in vitro drug screening data base. A recently developed program tracks the progress of events from acquisition of samples through the screening process, through the generation and distribution of test results. Furthermore, techniques have been developed to express and display data from the anticancer screen as "fingerprint" patterns. These patterns can demonstrate similarities between compounds related by either structural or biochemical mechanism of action.

The Pharmaceutical Resources Branch was responsible for the distribution of approximately one million units of investigational products to investigators world-wide during the past reporting period.

Detailed preclinical pharmacologic and toxicologic investigations have been conducted for nine anticancer compounds. Three pharmacology contracts were renewed. In addition, the Toxicology Branch continued two projects over the last year designed to provide more definitive information on the human toxicity investigational agents. These include studies on in vitro toxicity of new drugs in bone marrow committed stem cell compartments including a comparative hazard profile across species lines. Four antineoplastic agents were tested comparatively on murine and human committed stem cells.

An extensive program of extramural research is coordinated through the Grants and Research Contracts Operations Branch, NCI. The research spectrum encompasses biochemical, pharmacologic, and biologic research relevant to drug discovery and

development. Furthermore, the National Cooperative Drug Discovery Group program and the newly organized National Cooperative Natural Products Drug Discovery Group also will both contribute to innovative approaches to the treatment of malignancy and AIDS. This major investment of over 50 million in research support is invaluable in tapping the extensive talents of the extramural research community for the discovery of desperately needed new drugs agents for treatment of these fatal diseases.

Extensive basic intramural studies have been conducted to identify more selective approaches to chemotherapy including an evaluation of the interaction of growth factors with cells rather than on basic metabolic reactions such as those involved in nucleic acid synthesis. Projects have been initiated to develop new chemotherapeutic agents to block the action of growth factors. Nontraditional targets selected for drug design and study include: second-messenger inositol triphosphate and diacylglycerol; myristoylation of cellular oncogene products; protein kinase C; and selected G-proteins.

Two other extremely interesting compounds that have been developed within the intramural laboratories are cyclopentenyl nucleosides and 2-F zebularine. Phase I clinical investigations of the arabinosyl5-azacytosine (ara-AC) have been completed. This compound was synthesized in the intramural Laboratory of Medicinal Chemistry and currently has been evaluated through completion of Phase I trials in man in conjunction with pharmacokinetic studies. The agent is about to enter Phase II clinical testing in the extramural research program.

Extensive work has been conducted on the structure-activity relationships of an interesting series of compounds containing an unusual four-membered oxetane ring. A polypeptide laboratory has also been established to develop antisense polypeptides for potential clinical utility, and molecular modeling using critical structural parameters hopefully will result in additional active anticancer and anti-AIDS compound development.

Extensive investigation of camptothecin analogs as specific inhibitors of topoisomerase-I have been completed. Structural activity relationships of the various derivatives of camptothecin will be used to identify the analogs most likely to proceed into clinical development. While camptothecin is an agent that has previously had extensive clinical investigation, the development of specific derivatives with increased solubility without loss of potency in inhibition of topoisomerase-I may yield superior antineoplastic activity in the clinical setting.

Experiments are proceeding to determine how the selectivity of nitrogen mustard-like compounds can be improved by focusing attention on the major reaction site with DNA (DNA guanine-N7 positions). Finally, research continues in examining preferential repair mechanisms in DNA as a potential target for the development of new antineoplastic compounds.

B. Accomplishments in Antiretroviral Research

Anti-HIV Drug Screen. As of May 1989, over 18,000 synthetic compounds and 10,000 natural-product extracts have been tested in the anti-HIV drug screen. In addition to finding new active agents from chemical classes known to have anti-HIV activity, new active lead compounds and related derivatives have been discovered in 10 chemical classes not heretofore associated with the anti-HIV activity. The development

and implementation of assays for more detailed or mechanism-oriented biologic evaluations are continuing, and all new leads are being carefully evaluated.

Over the past year, the natural products chemistry effort has been focused on isolating and identifying naturally occurring compounds that possess anti-HIV properties. Extensive work has been conducted to obtain in pure form the individual compounds exhibiting the antiviral activity. Over the past year, two new classes of natural-product compounds (sulfolipids and phorbol ester analogs) that exhibit striking anti-HIV properties have been identified. These compounds are undergoing vigorous preclinical development.

Extensive cellular biochemical pharmacologic studies have investigated the metabolism of the dideoxynucleosides in cultured cell lines. Fluorinated dideoxynucleosides are resistant to decomposition by acid and as a consequence potentially will exhibit excellent oral bioavailability. Furthermore, fluorodideoxyadenosine is much more resistant to deamination by adenosine deaminase than is dideoxyadenosine. Finally, the deamination product, FddI, is completely refractory to phosphorylytic decomposition by purine phosphorylase. The data strongly indicate that FddI is a potentially very useful oral agent in the treatment of AIDS.

Extensive in vitro studies have been conducted to examine the effect of GM-CSF in combination with various anti-HIV nucleosides and virally infected monocyte/macrophages. While the data indicate the GM-CSF increases HIV-1 replication in monocyte/macrophages, the anti-HIV activity of AZT and related congeners in the cells may be explained by increased intracellular concentrations of the triphosphate anabolites of the AZT and D4T.

Detailed preclinical pharmacologic and toxicologic investigations have been conducted on five novel compounds demonstrating in vitro anti-HIV activity. A contract to identify agents with activity against the dihydrofolate reductase from Pneumocystis carinii was transferred to NIAID. Three new AIDS pharmacology contracts were initiated.

Seven new anti-HIV agents were tested comparatively on murine and human-committed bone marrow stem cells. An additional project investigated the comparative metabolism of anti-HIV agents in lymphocytes from experimental species and humans. The program directors are committed to expedite through the developmental pipeline unique promising new agents that might have potential benefit for patients with AIDS.

BIOLOGIC RESPONSE MODIFIERS PROGRAM (Associate Director, Dan L. Longo)

The Biologic Response Modifiers Program (BRMP) presents a comprehensive and unique program of both intra- and extramural basic and clinical research components charged with the responsibility of developing biological therapeutic agents that could lead to tumor regression in man. The program, which is located in Frederick, Maryland, is operationally well integrated. The intra- and extramural staff meet frequently to discuss strategies for biologic therapeutic development. Investigators from the program work in close collaboration with staff from DTP and CTEP to insure that the resources of the entire program are efficiently utilized to develop biologic agents in an efficient manner.

A. Accomplishments in Cancer Research

The extramural program accomplishments include the establishment of a number of promising contracts and grants covering a wide variety of potential therapeutic agents:

A significant effort to develop monoclonal antibodies as effective therapeutic agents in the treatment of melanoma, leukemia, colon cancer, renal cancer, and breast cancer is under way. The utilization of antibodies both with and without lymphokines to enhance the therapeutic effect will be examined.

Grants are in progress to enhance the delivery of therapeutic agents to tumors, to further understand the mechanism of action of cytokines and lymphokines, and to develop differentiation factors as potential therapeutic agents.

An extensive extramural clinical trial program has been established that will encompass both the early-testing and late-testing phases of these new therapeutic agents. Phase Ia/Ib clinical trials in the extramural community have been established to test monoclonal antibodies, immunoconjugates, cytokines, and other immunomodulators. Furthermore the Phase II and III testing capabilities have been established in the extramural community by working in close concert with CTEP.

A preclinical laboratory evaluation is operational at the Frederick Cancer Research Facility to examine methods of optimizing the dose intensity of chemotherapy programs in conjunction with biologic agents. Based on data generated from the preclinical evaluation laboratory, colony-stimulating factors are being interdigitated with chemotherapy in the treatment of Hodgkin's disease, and flavone acetic acid is being administered with IL-2. This laboratory is charged with the responsibility for the development of complex therapeutic interventions that employ combinations of both biologic and other types of agents accompanied with monitoring of both host and/or tumor response to these agents.

Intramural program accomplishments:

Extensive work has been continued to define factors that promote proliferation of B and T cells, and control mechanisms for growth arrest. CD3 complex of the T-cell receptor exists in two forms in adult T cells. One form is comprised of several chains plus a zeta-zeta homodimer. The other is comprised of several identical chains plus a zeta-eta heterodimer. CD3 molecules containing the eta chain are responsible for phosphoinositide turnover, and the zeta-zeta homodimer stimulates tyrosine kinase activity. Lymphokine production is a consequence of signal transduction as mediated by the zeta-zeta homodimer. Cellular autolysis, which is characterized by endonuclease destruction of the cell's DNA, requires phosphoinositide turnover and an intact eta chain. CD4 helper cells require signal transduction through the IL-2 receptor, through CD4, and need to encounter class II major histocompatibility antigens in the thymus in order to develop.

In addition to understanding the mechanisms of growth control in normal lymphocyte populations, studies are being conducted to elucidate the mechanisms of growth arrest of malignant B cells undergoing signal transduction to their surface immunoglobulin or other stimuli such as phorbol esters. The mechanism of lymphomagenesis may be actually the loss of expression of transforming growth factor-β receptors, and thus the loss of normal negative growth control. Furthermore, different signals can be used to produce growth arrest at distinct phases of the cell cycle. For example, anti-μ induces an arrest in G_1, while dolostatin induces a G_2/M block.

Preclinical studies in mice have demonstrated the potential utility of IL-1 to enhance survival following radiation and allogeneic bone marrow transplantation. Subsequently, the Clinical Research Branch is conducting a Phase Ia/Ib clinical trial of IL-1-α to evaluate the myeloprotective effect against radiation therapy and chemotherapy. The similarities of IL-1 and TNF have been demonstrated at the cellular level, including down-regulation of ornithine decarboxylase, induction of manganese superoxide dismutase, and phosphorylation of two intracellular proteins, p65 and p74.

IL-1 (and TNF) inducible leukocyte chemotactic inactivating factors have been characterized. The gene for IL-8, which is a monocyte-derived neutrophil, lymphocyte, and monocyte chemotactic factor, has been cloned. The gene maps to chromosome 4q12-21. Recombinant IL-8 has been produced. IL-8 induces margination and emigration of neutrophils, and its effects in vivo appear to mimic the capillary-leak syndrome seen with high-dose IL-2 therapy in man. IL-1 is necessary for the routine induction of IL-2 in mature T cells. IL-2 stimulates immature $CD4^-$, $CD8^-$ thymocytes to develop into mature $CD8^+$ gamma/delta T-cell receptor-expressing cells. In contrast, the same population of immature thymocytes exposed to IL-1 generates $CD4^+$ α/β T-cell receptor-expressing cells. Therefore, the possibility exists that IL-1 may be a useful way to expand $CD4^+$ T cells in patients with AIDS.

Transforming growth factor-β is a bifunctional regulator of hematopoiesis. Early bone marrow progenitors (and leukemic cells derived from early progenitors) were exquisitely sensitive to the antiproliferative effects of transforming growth factor-β. More mature precursors were insensitive to its growth-inhibitory effects. In contrast to its growth-inhibitory effects when used alone, the addition of transforming growth factor-β to GM-CSF greatly augmented marrow cell growth.

The gene has been cloned that appears to encode for the NK receptor necessary for binding between effector and target cell. One of the granular proteins thought to involved in cytolysis (natural killer cell cytotoxic factor) has been identified. Data has been generated that indicates that IL-2's effect on the large granular lymphocyte is mediated through the β-receptor. Furthermore, LGL's immature T cells have a common progenitor. There appear to be two subpopulations of LGLs; one representing the precursor for the LAK cell and one representing the precursor for NK cell. The subpopulations appear to be nonoverlapping.

Papillomavirus-infected cells, for example, cervical carcinoma cells, express GST-Pi in nearly 100% of the cases. An antibody to this isoenzyme is now undergoing investigation as a potential diagnostic reagent that could replace the PAP smears with an automated technique.

Effective doses of flavone acetic acid stimulate cytokine production including α-interferon, interferon-gamma, and tumor necrosis factor. The laboratory is attempting to identify if flavone acetic acid is synergistic with IL-2 in man.

The ras GTPase-activating enzyme (GAP) has recently been purified and cloned. GAP induced a rapid increase in diacylglycerol but no important increase in phosphoinositide turnover. In addition, the mechanism of action of cellular proteins that appeared to suppress the functions of the ras protein, including two small G proteins, are being examined with the potential for their being important for treatment strategies aimed at regulating ras expression. This has been implicated in over 90% of pancreatic cancers and the majority of colon cancers.

It has been demonstrated that the cytotoxic effects of TNF correlate with its capacity to induce a suicide enzyme that destroys DNA. This is a calcium-dependent endonuclease that breaks down the DNA of the cell.

Receptors for both interferon and IL-6 appear to be heterogeneous in cells of various linages. Tissue-specific post-translational modifications of the receptor may thus be responsible for the distinct biologic effects mediated by IL-6 in diverse cell lineages.

Intraperitoneal LAK cells plus IL-2 have been examined in patients with ovarian carcinoma or colon cancer limited to the peritoneal cavity. The response rate was 35%. There was an enormous pharmacologic advantage to administering the cells intraperitoneally. The dose-limiting toxicity was the development of adhesions that prohibited further delivery of therapy.

Long-term administration of α-interferon to patients with hairy cell leukemia has been piloted by the Clinical Research Branch. Continuous administration of interferon-α to 34 patients for a median of 3.5 years is a novel treatment strategy. All of the patients are continuing to respond to chronic interferon therapy, and evidence exists that some of the patients are continuing to improve through many years, with improvements in peripheral blood counts and a continued fall in the soluble IL-2 receptor levels. Toxicity associated with this approach is negligible.

In another study, alternating studies of deoxycoformycin and α-interferon were administered to 15 patients with hairy cell leukemia. Patients were treated for a total of 14 months and treatment was stopped. All patients responded to therapy. Because there were persistent hairy cells present in bone marrow, all were considered partial responders. All patients have remained stable for periods of up to three years off of therapy. One of these patients has persistent immunologic defects related to the deoxycoformycin; they do not appear to be prone to opportunistic infections.

The Clinical Research Branch has a wide variety of comprehensive programs to examine various cytokines alone and in combination with other biologic response modifiers and chemotherapy. A program to examine monoclonal antibody and immunotoxin therapy and a number of other innovative treatment strategies is currently proceeding very well.

B. Accomplishments in Antiretroviral Research

HTLV-I-infected B cells possess integrated HTLV-I provirus but express no viral message or proteins unless they are stimulated with phorbol esters. A B-cell gene product is capable of negatively regulating HTLV-I expression. Host regulation factors can affect HIV expression. Since viral latency is such a potentially important clinical problem, further investigation may lead to novel antiviral or viral therapies.

The HIV envelope protein (gp120) is a potent stimulator of the release of monokines (for example, IL-1 and TNF from monocytes). This protein interferes with leukocyte chemotaxis and function. Cytokines released upon exposure to this protein are capable of stimulating HIV viral infection and latently infected cells.

Interferon-gamma can down-regulate the expression of CD4 on monocytes, and therefore may help block this pathway of monocyte infection by HIV. Modulation of HIV transcription is another potential novel therapeutic strategy.

Chloroquine in vitro significantly alters the glycosylation of the gp120 envelope protein and results in the production of virions that are substantially less infectious. Therefore, inhibitors of glycosylation could result in therapeutic effect not only by reducing infectivity but by altering the gp120 effects on host defense.

Viral latency occurs by distinct mechanisms in various cell types. In T cells, viral latency appears to be related to host-cell DNA binding proteins that recognize the viral LTR and block expression. In monocytes, the virus has two forms of latency. In one form, virions are made but not extruded from the cell. The second form appears to involve methylation of the HIV viral LTR. These observations may provide yet another therapeutic target.

CANCER TREATMENT EVALUATION PROGRAM (Associate Director, Michael Friedman)

The Cancer Treatment Evaluation Program (CTEP) is responsible for the administration and coordination of the majority of the extramural clinical trials supported by DCT. These programs include the activities of the clinical cooperative groups, the Phase I and Phase II new-agent development contractors, and the whole investigator-initiated R01 and P01 grants relating to cancer treatment.

This program offers strategic input into the planning and implementation of disease-oriented treatment strategies across the spectrum of human malignancy. It also provides statistical consultation to extramural and intramural programs within DCT. The Regulatory Affairs Branch maintains close communication with the FDA, provides guidance in meeting the requirements of the federal regulations regarding the use of investigational agents, and monitors the conduct of clinical trials performed in NCI-sponsored clinical trials network.

Over the past year, staff from CTEP have been directly involved with many extremely important issues related to the success of clinical cancer research. In an effort to facilitate the access to effective new agents, interactions with the FDA have been enhanced by regularly scheduled meetings and bilateral efforts to expedite the drug development process. Efforts have been successful by the Investigational Drug Branch to improve the linkage with the pharmaceutical industry. These efforts are extremely important in the current era of concern for financial support of ongoing clinical research. Consequently, CTEP has taken a leadership role in directing attention to reimbursement as a major obstacle to clinical research. CTEP has sponsored meetings of representatives from the extramural research, pharmaceutical manufacturing, and lay communities to examine these complex issues.

CTEP and the Division of Extramural Activities (DEA) have been extremely concerned with the disproportionate impact of cancer on racial minorities. An ambitious program has been designed by the Clinical Investigations Branch to address these important concerns and to increase accrual from minority groups to cooperative group clinical trials. The cooperative groups system has continued to evolve into a highly efficient and productive resource for the advancement of the treatment of malignancy. Over the past several years there has been a marked increase in annual

accrual with the consolidation of cooperative groups resources. While the primary charge of the clinical cooperative groups is to perform high-quality therapeutic studies, there is a significant potential for performing important correlative scientific studies. Studies to examine such issues as prognostic factors, drug resistance, pharmacology, and clinical immunology are currently being exploited. The complete potential for utilization of the remarkable resources is as yet not fully realized. Staff from CTEP are working in close collaboration with other divisions (both DCPC and DCBD) to explore ways of increasing utilization of group expertise, patient populations, and data/specimen banks to answer critically important scientific questions.

Accomplishments for CTEP:

The Biometric Research Branch collaborated with investigators in the Developmental Therapeutics Program to carefully analyze the in vitro screening assay for both anticancer agents and anti-AIDS compounds. It was concluded that the cell line panels should include ten histologic subgroups and should have at least ten cell lines per subgroup.

The Biometric Research Branch has served as a primary statistician for several important clinical trials, including the staging and treatment of early ovarian carcinoma and a protocol comparing chemotherapy and radiation versus radiation in patients with residual non-small cell lung cancer. The results indicated that adjuvant chemotherapy was not appropriate for patients with very-early-stage ovarian carcinoma. Furthermore, the addition of chemotherapy with CAP and radiation in lung cancer studies demonstrated a modest survival advantage and greater time to recurrence advantage for patients treated with combined modality therapy rather than radiation therapy alone.

Efforts in statistical planning and methods research resulted in development of guidelines for the design and conduct of intergroup studies, careful examination of both subset and sequential analyses of clinical trials, and examination of sample size considerations for studies preparing survival curves using historical controls.

The Clinical Investigations Branch staff has organized strategic-planning meetings in selected disease sites to establish an overview of current therapeutic issues that may be resolved through collaborative clinical trials. Prevention of duplicative protocols and promotion of collaborative basic science studies has been a major emphasis of this Branch. Over the past year, 19 concepts for proposed major Phase III studies were reviewed, of which five went forward to become active studies.

By 1988, the Cooperative Group Clinical Trials Program had become markedly focused and more efficient. Since 1985, the number of groups has decreased from 18 to 11, with the number of studies decreasing from 585 to 490, while the absolute number of patient entries has signficantly increased to over 22,000 in the year 1988.

For the common adult malignancies, only 0.5 to 3% of available patients are actually studied per year on protocol. Since definitive studies may require over a thousand patients, an inordinate period of time to successfully complete and accrue patients to Phase III studies has been noted. With the dramatic expansion of basic and applied scientific research, there is an unprecedented number of important research options to pursue. Faced with this situation, in 1988 CTEP

organized a new initiative to increase accrual to cooperative group trials. Five protocols for four potentially curable diseases--adjuvant colon, rectum, bladder, and advanced lymphoma--were designated as "high priority" clinical trials of a national importance and were targeted for special attention. Accrual to all of these trials has been encouraging, and four of the five trials are accruing patients at approximately three times the projected rate. Six additional studies have been nominated for high-priority status for 1990 by cooperative group chairmen. The DCT Board of Scientific Counselors is examining these proposals prior to a decision upon endorsement. The proposed studies include three studies in breast cancer, one in non-small cell lung cancer, one in small cell lung cancer, and one in colon cancer.

In 1988 four strategy meetings were convened for esophageal carcinoma, multiple myeloma, advanced ovarian epithelial carcinoma, and head and neck cancer. Four additional sessions have been planned for 1989, including myelodysplastic syndromes, gastric carcinoma, non-small cell lung cancer and malignant melanoma.

Laboratory and clinical correlations are assuming an ever-increasing importance in the clinical research sponsored by the NCI through CTEP. Some of the more interesting laboratory and clinical correlations include studies of the biologic significance and genetic rearrangements in living cells of children with cancer. These studies offer increased reliability in correctly diagnosing patients and predicting which patients are likely to relapse. For example, n-myc oncogene copy number is directly related to clinical outcome in neuroblastoma. Peripheral neuroepithelioma is an uncommon tumor that is histopathologically indistinguishable from neuroblastoma. In fact, it is more closely related to Ewing's sarcoma. Patients with peripheral neuroblastoma treated as a Ewing's sarcoma do far better than those treated for neuroblastoma.

Immunophenotyping in conjuction with cytogenetic analysis permits determination of therapy for particular-risk subgroups of children with acute leukemia. Myeloma studies have demonstrated that B-2-microglobulin has been shown in several successful studies to be the single best predictor for outcome. In addition, in vitro correlates are being examined with respect to multidrug resistance (MDR) in treating patients with myeloma and non-Hodgkin's lymphoma with verapamil. Finally, immunophenotyping, cytogenetic data, and sophisticated molecular biologic studies are being correlated with clinical manifestations of acute myelogenous leukemia. Various subsets are being examined for their prognostic significance. The ultimate aim would be to improve the tailoring of therapy for various subsets of patients with acute leukemia.

There are over 126 investigator-initiated funded grants and contracts in the portfolio of the Clinical Investigations Branch. Recently, an RFA has been issued for investigating important correlations between drug treatment and resistance that hopefully will provide innovative approaches to overcoming drug resistance and improvement in cancer treatment.

Several remarkable individual achievements include Dr. William McGuire's demonstration at the Univ. of Texas, San Antonio, of a positive correlation of the diploid, low S-phase tumors status with diseasefree survival for patients with node-negative breast cancer. Furthermore, he has demonstrated cathepsin-D protein in breast cancer samples indicates an important predictor of disease-free and overall survival for node-negative patients.

Dr. Douglas Torme, at the Univ. of Wisconsin, has had a remarkably high complete remission rate in patients with breast cancer who were being treated with chemotherapy and long-term tamoxifen administration.

Dr. William Neuwelt, at the Univ. of Oregon, afforded a significant prolongation of the survival in patients with primary central nervous system lymphoma. The patients are treated with combination chemotherapy in association with an osmotic blood-brain barrier disruption technique.

Dr. Ray Warrell at Memorial Sloan-Kettering Cancer Institute has demonstrated the benefit of restoring bone mass by the therapeutic intervention with gallium-nitrate in patients with multiple myeloma.

Dr. James Speyer at New York University reported on the cardioprotective effect of ICRF-187 against adriamycin cardiotoxicity.

Finally, one of the most exciting clinical trial observations over this past year has been the demonstration of a statistically significant benefit for patients with Dukes' C adenocarcinoma of the colon to receive adjuvant therapy with 5-fluorouracil and levamisole. This combination has improved the disease-free interval and also the number of recurrences in the treated group. A recent intergroup study has been analyzed on an interim basis and appears to substantiate findings of the NCCTG. The NCI is in the process of issuing a clinical announcement regarding this finding. Since colon cancer is such a frequent type of malignancy, this significant observation may have benefit for a large number of patients with micrometastatic disease.

In addition to many other high scientific priorities, CTEP will be focusing on protocols with direct attention towards increased cancer mortality seen among under-served minority populations. Clinical trials will be sought in diseases with a disproportionate incidence in mortality in minorities (e.g., multiple myeloma, esophageal carcinoma). Inappropriate obstacles to the participation of the elderly in clinical trials have been removed.

The Clinical Trials Cooperative Group will be encouraged to become a resource for the entire NCI effort in the conduct of trials in cancer prevention, etiology, and biology.

Various biologic agents are being examined in combination; for example, alpha-interferon and gamma-interferon are being studied in combination for chronic myelogenous leukemia.

IL-2 is being investigated in combination with chemotherapy (cisplatin); combinations of IL-2 with adriamycin and and cyclophosphamide are also being pursued.

Combination cytokine regimens are being explored, including IL-2 in combination with either α-interferon or gamma-interferon, and IL-2 in combination with tumor necrosis factor.

Extensive studies to investigate colony-stimulating factors (G-CSF and GM-CSF) in combination with intensive chemotherapy and bone marrow transplantation and in the setting of refractory acute leukemia in an effort to bring cells into cycle, and, therefore, make them more responsive to standard cell-cycle-specific chemotherapy are in progress.

Extensive work has been done to make several promising new agents available through group-C/treatment IND mechanisms for the following clinical situations:

Treatment	Disease-Specific Indication
Levamisole in combination with 5-FU	Adjuvant treatment for Dukes' C adenocarcinoma of the colon
VM-26 in combination with ara-C	Refractory acute lymphoblastic leukemia
Deoxycoformycin	Hairy cell leukemia
Fludarabine monophosphate	Protocol in preparation for chronic lymphocytic leukemia

Phase I trials have been completed in fazarabine, flavone acetic acid, HMBA, piroxantrone, and merbarone.

Two agents that have completed Phase I testing have demonstrated encouraging activity in initial Phase II testing. Amonafide has produced objective responses in Phase II trials in patients with breast cancer. Taxol has demonstrated a response rate of 33% in patients with refractory ovarian carcinoma.

Trimetrexate continues to produce excellent responses against Pneumocystis carinii pneumonia in patients with AIDS.

Carboplatinum has been approved by the FDA for the second-line treatment of ovarian carcinoma.

Suramin, which is an agent that had been used for treating trypanosomiasis since 1920, has demonstrated evidence of activity in adrenal carcinoma, renal carcinoma, and prostatic carcinoma. Blood-level monitoring of the plasma concentration is extremely important to avoid one serious potential toxicity (neurotoxicity). Phase II confirmatory trials are being initiated in the extramural community.

The data on fludarabine monophosphate is being prepared for submission to the FDA for marketing approval in the treatment of chronic lymphocytic leukemia. The development of this agent represents a major achievement for the NCI. The NCI (CTEP) has been largely responsible for the successful completion of Phase I and Phase II studies with this active new agent.

Two compounds that were selected for activity in the human tumor colony-forming assay have reached Phase I trials. The first compound chosen for clinical trials based on the activity in this assay was dihydrolenperone; Phase I trials in lung cancer patients have been initiated. Chloroquinoxaline sulfonamide, which is the second compound with outstanding activity in this assay, has been selected for clinical development.

An electronic clinical drug request system has been developed that will markedly reduce the time to investigational drug delivery to investigators. The procedure was appropriately piloted and was found to be very efficient. This innovation will allow NCI to reduce cost by obviating the need for large drug inventories to be maintained within the individual investigational off-site extramural programs.

The total number of drug orders over the past year to the Investigational Drug Branch (IDB) was 15,504. There were 696 special exceptions; 550 patients received drugs under the group C mechanism; and there were over 1.2 million containers distributed over the past year to investigators in the intra- and extramural programs. Therefore, the magnitude of the task accomplished by IDB in supplying new agents to investigators can be appreciated.

Thirty-five INDs for cytotoxic and biologic anticancer and anti-AIDS agents were prepared and submitted to the Center for Drug Evaluation and Research and the Center for Biologic Evaluations and Research of the FDA.

INDs for ten agents were discontinued.

Meetings were initiated with the Division of Antiviral Drug Products of the FDA to determine the preclinical data and the IND format required for agents used to treat patients with AIDS.

Procedures were implemented for monitoring limited multi-institutional Phase I studies carried out by the cooperative groups. Five cooperative groups are currently conducting Phase I trials.

Special audits were performed to examine the data and verify response determinations in promising Phase II trials, including dibromodulcitol in ovarian carcinoma, suramin in prostate cancer, high-dose carboplatinum in ovarian cancer, and α-interferon with 5-FU in colon cancer.

In summary, the broad scope of research activities within the Division of Cancer Treatment encompasses both basic and clinical research involving all modalities of cancer therapy. The resources of the intramural and extramural investigators are coordinated through the scientific leadership of the Office of the Director and the Associate Directors of each of the five programs. The ultimate goal of this program is to translate scientific discovery into significant improvement in survival and, ultimately, cure of the patients with cancer.

PUBLICATIONS (OD-DCT)

Chabner BA, Browne MJ. Prospects for the drug treatment of cancer. In: Maulitz RC, ed. Transactions and Studies: Cancer, Trauma, and Cardiovascular Disease. Philadelphia: College of Physicians, 1988;103-109.

Vogelzang NJ, Chabner BA, Periman P, Ohrt DK, McCann BC, Burns JM, Bayer R. Reimbursement issues in clinical oncology. Semin. Oncol. 1988;15:34-43.

Chabner BA. Editorial. In celebration of a Nobel Prize. JNCI 1988;80:1512-13.

Glick JH, Chabner BA, Benson JA Jr. Evaluation of clinical competence in medical oncology training programs. J. Clin. Oncol. 1988;6:1516-19.

Chabner BA, Shoemaker D. Drug development for cancer: implications for chemical modifiers. Int. J. Radiat. Oncol. Biol. Physics 1989;16:907-9.

Rothenberg M, Ling V. Multidrug resistance: molecular biology and clinical relevance. JNCI 1989;81:907-10.

Chabner BA, Fojo AT. Editorial. Multidrug resistance: P170, with its allies, proves an elusive foe. JNCI 1989;81:910-13.

Rothenberg M, Wilson W, Chabner BA. Complications of drug therapy for cancer: general review. In: Plowman PN, McElwain TJ, Meadows AT, eds. The Complications of Cancer Management. Surrey, UK: Butterworth, 1989;in press.

Chabner BA, Horwitz SB. Plant alkaloids. In: Pinedo HM, Longo DL, Chabner BA, eds. Cancer Chemotherapy and Biological Response Modifiers, Annual 11. Amsterdam: Elsevier, 1989;in press.

Rothenberg M, Johnson G, Laughlin C, Green I, Craddock J, Sarver N, Cohen JS. Oligodeoxynucleotides as antisense inhibitors of gene expression: therapeutic implications. JNCI;in press.

Shibley L, Brown M, Schuttinga J, Rothenberg M, Whalen J. Cisplatin in the treatment of advanced stage testicular cancer: cost savings analysis. JNCI;in press.

Definitions of Contract Groupings
FY1989 Annual Report

Drug Development

Compound Discovery - Preclinical contract program focusing on the discovery and screening of potential anticancer agents. Includes both natural products and synthetics, as well as testing (screening) *in vitro* and *in vivo*.

Compound Development - Includes data management, pharmacology and pharmacokinetics testing, formulation and analytical profiles, and toxicological protocols to meet FDA requirements.

Clinical Support - Preclinical contracts which provide direct support to the clinical trials program (excluding purchases).

Biologics Development

All non-clinical contracts administered by the Biological Response Modifiers Program, excluding contracts in support of the intramural program.

Diagnostic Imaging

Contracts administered by the Diagnostic Imaging Research Branch, RRP.

Radiation Development

Preclinical radiation contracts, including screening and synthesis of radiosensitizers and radioprotectors, dose calculations, and other preclinical radiotherapy contracts.

Clinical Trials

Drug Evaluation - Phase I, II, III drug development contracts administered by the Cancer Therapy Evaluation Program, including foreign clinical contracts in support of FDA requirements.

Biological Evaluation - Task order contracts for Phase I/II clinical trials of BRM's.

Radiotherapy - All clinical radiotherapy contracts administered by the Radiation Research Program.

Other - Contracts which support other research requirements throughout the Division of Cancer Treatment, including program support, data management for extramural contracts, and other technical support. Does not include intramural support contracts.

Support to Intramural

Contracts which directly support intramural research activities in the Developmental Therapeutics Program, the Clinical Oncology Program, and the Biological Response Modifiers Program.

Drug Purchases

The purchase of investigational agents used in DCT-sponsored Phase I/II/III clinical trials, where DCT provides the agents being used in the trial to both extramural and intramural investigators.

Program Management

Includes administration and dissemination of information to the medical and scientific community.

TABLE I

DCT Contract Program for FY 1989
(Dollars in Thousands)

		TOTALS
I.	Drug Development	$53,162
	A. Compound Discovery - Subtotal	35,363
	1. Acquisition	11,922
	a. Natural Products	6,900
	b. Synthesis	5,576
	2. Screening	23,441
	a. In Vivo	8,761
	b. In Vitro	9,120
	c. Screening Support	5,996
	B. Compound Development	3,244
	C. Clinical Support	9,174
	D. AIDS Drug Development	4,391
II.	Biologics Development	1,720
III.	Diagnostic Imaging	177
IV.	Radiation Development	992
V.	Clinical Trials - Subtotal	13,752
	A. Drug Evaluation (Phase I/II/III)	6,567
	B. Biological Evaluation (Phase I/II)	3,505
	C. Radiotherapy	2,113
	D. Other	1,669
VI.	Support to Intramural	892
VII.	Drug Purchases	3,450
	A. Cancer Drugs	2,250
	B. AIDS Drugs	1,200
VIII.	Program Mangement	458
	TOTAL DCT CONTRACTS	$74,705

TABLE II

DESCRIPTION OF CONTRACTS

IN THE

DIVISION OF CANCER TREATMENT

OFFICE OF THE DIRECTOR

JAPANESE FOUNDATION FOR CANCER RESEARCH (N01 CM3-6011)

The objective of this contract is the maintenance of a chemotherapy liaison office at the Japanese Foundation for Cancer Research (JFCR) in Tokyo, to provide up-to-date information services in support of our cancer treatment program, both preclinical and clinical. The contract also serves as a liaison to acquire pure natural products for our screening programs. Recently, a training program has been established between NCI and JFCR for the bilateral exchange of postdoctoral fellows between the U.S. and Japan. This is a cost-sharing contract and is strongly supported by the JFCR and the Japan Society for the Promotion of Science.

TECHNICAL RESOURCES, INC. (N01 CM5-7658)

This contract provides technical support services to the Office of the Director, as well as to the program areas of DCT in the performance of the planning and analytical tasks, and general logistical support in the development of related or otherwise required documentation and conference support activities of the Division. This contract was awarded in April 1985 for a five-year period, and is currently being recompeted.

U.S. DEPARTMENT OF STATE (YO1-CM5-0135)

Through an interagnecy agreement between the Department of State and the Department of Health and Human Services, the NCI (DCT) operates a liaison office in Brussels, Belgium. This office is primarily responsible for coordinating the NCI drug discovery and development program in Europe in close collaboration with the European Organization for Research and Treatment of Cancer (EORTC) and the Cancer Research Campaign, (CRC, UK). The Brussels office also plays a key role in facilitating the NCI-EORTC Fellowship Program which promotes exchanges between European investigators and the NCI.

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

ALABAMA, UNIVERSITY OF (N01-CM4-7679)

This contract supports a Phase Ib study of the murine monoclonal antibody (14.G2a) against the GD2 sialoganglioside antigen in patients with malignant melanoma, alone and in combination with interleukin-2 (IL-2). The objectives of this study are: (1) to determine whether different dose levels of IL-2 in combination with monoclonal antibody will enhance antibody directed cellular cytotoxicity in peripheral blood monocytes and lymphocytes; (2) to assess *in vitro* immune activation by different IL-2 doses as measured by circulating mononuclear cell phenotypes and cytotoxic activity and by serum levels of interferon- ; (3) to assess the effects of IL-2 on the development of human anti-mouse antibody and anti-idiotype responses; and (4) to record toxicity and antitumor effects of the regimen. Antibody is currently in production for this contract, and the contractor is performing preclinical evaluation of the antibody in preparation for beginning the clinical trial. This contract was due to expire in September of 1988; however, due to a shortage of 14G2a antibody, it was late in getting started. It is estimated that this contract will now run until September of 1990.

ALABAMA, UNIVERSITY OF (N01-CM9-7611)

This clinical contract supports the design and conduct of Phase I/II clinical trials of monoclonal antibodies, immunoconjugates and other targeting agents alone or in combination with other investigational or standard therapy. This is a shared study between the Biological Resources Branch and the Cancer Therapy Evaluation Branch in the Division of Cancer Treatment. These trials will focus on studies of mechanisms of antitumor response and immune modulation. Current areas of emphasis include the elucidation of complex interactions between the biological agent(s), the host effector cells and the tumor which may lead to tumor regression. Thus, antibody directed cellular cytotoxicity, tumor infiltrating lymphocyte activation, and the induced inflammatory response will be under scrutiny. In addition, detailed studies will be performed of pharmacokinetics and biodistribution, which are intended to lead to better application of monoclonal antibodies to deliver radionuclides, toxins and drugs to tumor cell targets. Of special interest will be the influence on these processes of specific modifications of monoclonal antibodies, such as changes in affinity, isotype, chimerization, and substitution of F(ab')2 fragments. This is a cost reimbursement contract which was awarded March 31, 1989 and will expire on March 30, 1994.

CALIFORNIA, UNIVERSITY OF (N01-CM4-7682)

This contract supports a Phase Ib study of effects of intralymphatic *vs.* intravenous injection of interleukin-2 (IL-2) on the numbers and types of lymphocytes circulating through the thoracic duct. Preliminary investigations by this group have generated lymphokine activated killer cells from thoracic duct lymphocytes. The objectives of this study are: (1) to study activation of thoracic duct lymphocytes *ex vivo*; (2) when

activation conditions have been optimized, to study reinfusion of cells with IL-2 for cancer treatment; (3) to monitor patients for toxicity, immunological parameters, and clinical antitumor response.

Another segment of this contract is the Phase Ia/Ib study of granulocyte-monocyte colony stimulating factor (GM-CSF), comparing biological and therapeutic effects of intravenous vs. subcutaneous administration, with and without cyclophosphamide to inhibit suppressor lymphocyte activity. The objectives of this study are: (1) to assess monocyte/macrophage maturation, activation, cytotoxicity, chemotactic response, and cytokine production; (2) to assess hematopoietic maturation in peripheral blood and bone marrow, GM-CSF pharmacokinetics, anti-GM-CSF antibody formation, peripheral blood lymphocyte phenotype, and granulocyte maturation and activation; and (3) to record toxicities and clinical antitumor response. This contract is scheduled to expire on September 30, 1989.

CALIFORNIA, UNIVERSITY OF (NO1-CM8-7289)

This clinical contract supports the design and conduct of Phase I/II clinical studies of cytokines and immunomodulators. These studies focus on elucidation of specific mechanisms involved in the immune modulation and antitumor responses associated with biologic agents, alone or in combination with other modalities. This is a cost reimbursement contract which was awarded September 30, 1988 and expires on September 29, 1993.

CLEVELAND CLINIC FOUNDATION (NO1-CM4-7673)

This Phase Ib study is designed to explore the optimal schedule for the administration of clonally expanded tumor infiltrating lymphocytes (TILs) with 5(TIL: 1-5 x 10^9 cells i.v. D1,8) using multiple dose levels of recombinant interleukin-2 (rIL-2) (Hoffmann-LaRoche: 0-6 x 10^6 u/m^2/24 hrs i.v. D1-5, D8-12) in patients with metastatic renal cell carcinoma and minimal tumor burden. The objectives of this study are: (1) to study the toxicity of TILs and rIL-2 given as a constant infusion compared with lymphokine activated killer cells (LAK) and rIL-2; (2) to study conditions influencing growth and lytic activity of TILs; (3) to characterize the effector cell population in clonally expanded TIL compared with cytolytic potency to LAK cells from the peripheral blood; (4) to record objective tumor responses in this regimen. The clinical trial is underway.

Another segment of this contract is the Phase Ib study of macrophage activation by granulocyte-monocyte colony stimulating factor (GM-CSF) in patients with lung cancer. The objectives of this study are: (1) to evaluate the activation of alveolar and tumor-associated macrophages, in comparison with those in the peripheral blood or other tissues. Patients will undergo serial pulmonary lavage and tumor biopsy for assessment of macrophage activation and cytotoxicity; (2) to determine clinical toxicities and record antitumor responses; and (3) to study hematopoietic maturation in the blood and bone marrow, GM-CSF pharmaco- kinetics, induction of anti-GM-CSF antibody, and changes in cutaneous T-cell lymphoma and natural killer activity. This contract is scheduled to expire on October 31, 1989.

CLEVELAND CLINIC FOUNDATION (NO1-CM9-7622)

This clinical contract supports the design and conduct of Phase I/II clinical studies of cytokines and immunomodulators. These studies focus on elucidation of specific mechanisms involved in the immune modulation and antitumor responses associated with biologic agents, alone or in combination with other modalities. This is a cost reimbursement contract which is scheduled to run until FY 1994.

DAMON BIOTECH, INC. (N01-CM6-7873)

The purpose of this contract is to produce large-scale quantities of clinical-grade monoclonal antibodies. The contractor is expected to produce upon receipt of a given murine hybridoma or murine cell line producing a mouse/human chimeric antibody, 10 to 50 grams of the antibody by in vitro culture techniques. Following production of the bulk monoclonal antibody, the contractor will perform the necessary purification, determine the isotype, perform mouse antibody production testing and finally vial and label the preparation. All procedures will comply with the Good Laboratory Practice and Good Manufacturing Practice procedures as defined by the Food and Drug Administration. The preparation of a Drug Master File for each monoclonal antibody produced, which can be accessed by Biological Response Modifiers Program-sponsored clinical investigators for the purpose of aiding in the filing of an investigational new drug, is an important responsibility of the contractor. This contract is scheduled to expire August 31, 1989.

FRED HUTCHINSON CANCER RESEARCH CENTER (N01-CM4-7668)

This Phase Ib clinical trial is designed to evaluate a murine monoclonal antibody (MG21/22) reactive with the sialoganglioside, GD3, which is found on the cell surface of most melanoma cells. The objectives of this study are: (1) to determine pharmacokinetics, clinical toxicity, and immunological response monitored in an initial dose-finding study of monoclonal antibody alone; and (2) to perform a successor study which will evaluate monoclonal antibody in combination in interleukin-2. This clinical trial began with the monoclonal antibody MG21. The occurrence of severe, unexpected adverse reactions in the first two patients treated at the highest dose level prompted suspension of the study. It now appears that these reactions were due to a bad batch of MG21 monoclonal antibody. The study has reopened with MG22, a related and improved murine monoclonal antibody.

Another segment of this contract supports a preclinical and Phase Ib study of interleukin-4 (IL-4), interleukin-2 (IL-2) and anti-CD3 monoclonal antibody for cytolytic effector cell generation. The objectives of this study are: (1) to optimize conditions for ex vivo augmentation of effector cell activity with IL-2/IL-4/monoclonal antibody in patients receiving IL-2 in an IL-2/lymphokine activated killer trial; and (2) to perform a Phase Ib clinical trial of IL-2/IL-4/monoclonal antibody + effector cells using that methodology. Preclinical studies are underway, and the clinical trial is awaiting completion of IL-4 production. This contract is scheduled to expire on September 30, 1989.

HAZLETON LABORATORIES, INC. (N01-CM7-3710)

The purpose of this contract is to provide effective inventory, distribution and quality assurance confirmation for biological response modifiers. The contractor is responsible for receipt, dispensing, storage, distribution, and inventory control of biological agents. Quality assurance evaluation involves specific assays to confirm sterility and assays to determine pyrogenicity and endotoxin levels. The contractor performs general safety tests for biological agents intended for clinical use in compliance with Government regulations and helps in the development of master files and investigational new drugs for biologics. Currently, the contractor provides for storage and distribution of approximately 100 different biologics. The contractor manages a repository distributing agents to qualified intramural and extramural investigators for preclinical studies. The contract also provides for ascites production and purification of monoclonal antibody and has produced monoclonals specific for melanoma, colon, breast cancers, T-cell receptor, and lymphocyte antigens. The contractor also performs, under Good Medical Practice conditions, chemical linkage of chelate to allow radionuclide labelling of monoclonal antibodies. This contract was awarded in September 1987 and will expire September 9, 1992.

HYBRITECH, INC. (N01-CM6-7718)

This contractor performs coupling of chemotherapeutic drugs, toxins and radioisotopes to monoclonal antibodies directed against specific antigens found on human tumor cells. Appropriate tests are carried out on conjugates to demonstrate that the cytotoxic agent-antibody conjugates retain antigen-antibody specificity comparable to the unmodified antibody and cytotoxicity in excess of the nonderivatized cytotoxin. The contractor is required to scale up the appropriate conjugation procedure to provide sufficient quantities of a human use product for preclinical and preliminary clinical trials. The contractor is conjugating pseudomonas exotoxin to the monoclonal antibody OVB-3 for intramural National Cancer Institute trials. Experiments have been ongoing to couple methotrexate, ricin A chain, Yttrium-90, Indium-111 and Iodine-131 to T101, an antibody directed against a human T-cell differentiation antigen, and 9.2.27, an antibody directed against a human melanoma cell antigen. The contractor has been supplying T101 conjugated to indium and iodine for biodistribution, imaging and therapy studies. The contractor continues to refine the T101-methotrexate conjugate, specifically reducing the level of liver localization by conjugating reagents with a lower level of activated chelating agents. This contract expired March 1, 1989.

HYBRIZYME CORPORATION (N43-CM9-7605) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract is to develop a computerized Monoclonal Antibody Cancer Data Bank to allow investigators rapid access to current information on monoclonal antibodies for use in the diagnosis and therapy of cancer. In Phase I, a prototype will be developed containing information on representative monoclonal antibodies. This is a fixed price contract with an expected duration of six months and runs from February 1, 1989 to July 31, 1989.

IMMUNOMEDICS, INC. (N44-CM8-7778) (SMALL BUSSINESS INNOVATION RESEARCH Program)

This contract studied the use of Tc-99m labeled NP-4 monoclonal antibody for radioimmunodetection of human colon cancers transplanted into the livers of nude mice. Optimal methods for radiolabeling with Tc-99m were developed in Phase I. Phase II further studied the kinetics of tumor localization, imaging quality and catabolism. It is hoped that the development of Tc-99m conjugated monoclonal antibody for imaging will be useful since the capability of performing Tc-99m scans is already widely available in nuclear medicine departments. This contract expired May 31, 1989.

JEFFERSON MEDICAL COLLEGE (N01-CM6-7902)

This is a Phase Ib clinical study that will involve 16 patients with advanced sarcomas which are refractory to standard therapy. The objectives of the study are to assess the toxicity, therapeutic efficacy, and modulation of immune parameters with tumor-specific cytotoxic T-lymphocytes ("educated" lymphokine activated killer (LAK) cells). These LAK cells will be generated by incubation of leukapheresed peripheral blood lymphocytes with autologous tumor and interleukin-2 (IL-2) ex vivo. The regimen consists of leukapheresis on days -7, 0, and +4, and ex vivo incubation of Ficoll-separated cells with IL-2 and autologous tumor for 7 days. Cyclophosphamide (300mg/m2) is given intravenously on Day 3 to reduce host suppressor cells. Reinfusion of "educated" LAK is given on days 0,7, and day 11 with IL-2 (Hoffmann-LaRoche): 75,000 u with 3000 U/kg/hr by continuous intravenous infusion x 72 hours. The three populations of lymphocytes, removed from the patient at different points in the therapeutic course, will be compared in immunological function and ability to be "educated" to react against autologous tumor cells. Patient accrual is underway. This contract is scheduled to expire on November 29, 1989.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (N01-CM8-7270)

This clinical contract supports the design and conduct of Phase I/II clinical studies of cytokines and immunomodulators. These studies focus on elucidation of specific mechanisms involved in immune modulation and antitumor responses associated with biologic agents, alone or in combination with other modalities. This contractor is currently performing studies of marrow colony stimulating factors, with specialized assays to clarify their effects on the proliferation and maturation of hematopoietic cells. This is a cost reimbursement contract and expires on September 29, 1993.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (N01-CM9-7609)

This clinical contract supports the design and conduct of Phase I/II clinical trials of monoclonal antibodies, immunoconjugates and other targeting agents alone or in combination with other investigational or standard therapy. These trials will focus on studies of mechanisms of antitumor response and immune modulation. Current areas of emphasis include the elucidation of complex interactions between the biological agent(s), the host effector cells and the tumor which may lead to tumor regression. Thus, antibody directed cellular cytotoxicity, tumor infiltrating lymphocyte

activation, and the induced inflammatory response will be under scrutiny. In addition, detailed studies will be performed of pharmacokinetics and biodistribution, which are intended to lead to better application of monoclonal antibodies to deliver radio- nuclides, toxins and drugs to tumor cell targets. Of special interest will be the influence on these processes of specific modifications of monoclonal antibodies, such as changes in affinity, isotype, chimerization, and substitution of F(ab')2 fragments. This contract is shared between the Biological Resources Branch and the Cancer Therapy Evaluation Branch in the Division of Cancer Treatment. This a cost reimbursement contract awarded in September 30, 1988 and will expire on September 29, 1993.

MEMORIAL SLOAN-KETTERING CANCER CENTER (N01-CM4-7665)

This Phase Ib clinical trial uses R24 monoclonal antibody combined with interleukin-2 (IL-2), in patients with metastatic malignant melanoma. Monoclonal antibody R24 is directed against GD3, a sialoganglioside expressed in large amounts on the surface of malignant melanoma cells. R24 also cross-reacts with a lymphocyte surface antigen and enhances effector cell function. R24 has shown clinical responses when given alone. This clinical study used a fixed dose of IL-2 (Cetus; 10^6u/m^2/day on days 1-5 and days 8-12) uses plus one of four dose levels of R24 up to 12 mg/m^2 IV on days 8, 12. The objectives of the trial design are to evaluate toxicities, biological and antitumor effects. Of 21 patients treated, there have been 1/21 PR and 2/21 MR. A significant increase in total lymphocyte count was seen during treatment compared to baseline pretreatment values, with a suggestion that the degree of lymphocytosis was increased at higher dose levels of R24. Significant increases were also seen in CD3+, CD4+, CD8+, CD16+, HLA-DR and IL-2R+ PBLs. Activation of natural killer and lymphokine activated killer activities were seen. T-lymphocytic (T3+, T8+, Ia+) infiltrates were increased in tumor biopsies compared to pretreatment biopsies. Toxicities were characteristic of IL-2. Dose limiting toxicity was not reached at the highest dose of monoclonal antibody (12 mg/m^2/day on days 8-12) and IL-2. Analysis of the data is continuing.

Another segment of this contract is a Phase Ib clinical trial designed to evaluate treatment with various dose levels of R24 $F(ab')_2$ fragments in patients with metastatic melanoma. The R24 monoclonal antibody reacts with a surface antigen on some T-lymphocytes enhancing effector cell activity. This may be the mechanism underlying observed antitumor effects following the administration of low doses of intact R24. If so, use of the $F(ab')_2$ fragments may allow the antitumor effect to be preserved while eliminating the effects of the murine Fc portion. The objectives of this study are: (1) to evaluate toxicity and antitumor effects at different dose levels of R24 $F(ab')_2$; (2) to assay titers of free $F(ab')_2$, absolute levels in serum after injection. Assay for anti-mouse immunoglobulin and anti-idiotype responses; and (3) to monitor antibody directed cellular cytotoxicity and the absolute number and percentage of GD3+ T-lymphocytes in peripheral blood during treatment, and activation of T-cells before and during treatment. Opening of the trial is awaiting completion of R24 production currently underway. This contract is scheduled to expire on June 29, 1990.

NEORX CORPORATION (N01-CM5-7719)

The purpose of this contract is to develop a centralized, coordinated program for uniform preclinical testing and evaluation of monoclonal antibodies and their immunoconjugates prior to entry into clinical trials. The contractor will test and evaluate monoclonal antibodies and immunoconjugates in several test systems: (1) immunoreactivity against a panel of known tumor cells to define relationships with other monoclonal antibodies and establish epitope reactivities by molecular or serologic means; (2) in vitro cytotoxicity assays; (3) virus testing for lymphocytic choriomeningitis, retrovirus and mouse antibody production test; (4) immunohistologic screening to define antigen positive tissues and specificity; (5) antitumor effects in the nude mouse model and subrenal capsule assay; and (6) animal toxicity evaluation will be evaluated at each level of testing and must exceed pre-established standards of specificity and activity before proceeding to the next level of evaluation. Monoclonal antibodies successfully progressing through the screening process will then be considered for clinical evaluation. Monoclonal antibodies against a 250 kilodalton human melanoma antigen, against human melanoma sialogangliosides and against human colon carcinoma have been evaluated. In addition, monoclonal antibodies conjugated with Pseudomonas exotoxin have been investigated for cytotoxicity in vitro. This contract was awarded in FY 1986 and expired on January 1, 1989.

NEW YORK UNIVERSITY (N01-CM6-7895)

This Phase Ia/Ib clinical trial will be conducted to assess effects on macrophage activation and cytotoxicity of various dose levels of granulocyte-monocyte colony stimulating factor (GM-CSF). The objectives of this study are to determine clinical toxicities, antitumor response, hematopoietic maturation in the blood and bone marrow, GM-CSF pharmacokinetics, and induction of anti-GM-CSF antibody. Protocol accrual is underway. This contract is scheduled to expire on October 31, 1989.

OHIO STATE UNIVERSITY (N01-CM4-7666)

This Phase Ib clinical trial in metastatic renal cell cancer is designed to explore an alternative methodology using phenylalanine methyl ester for ex vivo production of lymphokine activated killer (LAK) cells from patients being treated with interleukin-2 (IL-2) ("expanded LAK"). In the trial design, IL-2 (Cetus) is given 3 MU/m^2 by C.I. days 1-5. Leukapheresis is performed days 8-11. LAK cells are cultured and expanded for 10 days. LAK cells and IL-2 (3 MU/m^2) are given at days 18-21. The objectives of the trial are: (1) to determine toxicity and record antitumor effects; (2) to correlate LAK activity against tumor, LAK cell phenotype, and disease histology with response; and (3) to perform imaging studies with ^{111}In-labeled LAK cells in selected patients. Protocol accrual is underway in renal cell patients. The trial will be extended to melanoma patients. This contract is scheduled to expire on January 30, 1990.

PITTSBURGH CANCER INSTITUTE (N01-CM4-7681)

This contract supports a Phase I clinical trial of multiple dose level combinations of interferon-alpha (IFN-α) (Hoffmann-LaRoche; 2.5, 5, 10 or 20 MU/m^2) and interferon-gamma (IFN-) (Biogen; 30, 300 or 1000 mcg/m^2) in patients with renal cell carcinoma. Objectives of this study are: (1) to determine pharmacokinetics of the study agents; (2) to evaluate immune modulation under this regimen; and (3) to determine optimal biological doses of these agents in combination; to record toxicities and antitumor responses. Thirty-six patients have been accrued, and 30/36 had prior nephrectomies. The doses reached were 20 MU/m^2 of IFN-α QD x 7, and 300 mcg/m^2 of IFN- QD x 5 every third week. At this level, cumulative hematologic toxicity has necessitated dose reductions. Grade 4 neutropenia has been seen in some patients at doses above 300 mg/m^2 of IFN- , irrespective of the IFN-α dose. Cardiac toxicity was prominent at the highest dose levels (V-tach, CHF, cardiovascular collapse and death). Thirteen/35 patients are on maintenance therapy: 2CRs, 4/36 PRs, 7SD/MR. CR/PR durations have ranged from 2-16+ months. No responders had primary tumor intact. Analysis of immunological data suggests that combined treatment with rIFN- and rIFN-α resulted in a greater increase in NK-activity than rIFNα alone (3/15 vs 6/17). If natural killer-activity is considered as an indication of the optimal biologic dose, the data suggests that rIFN-α at 2.5-5 MU/m^2 combined with 300-1,000 mcg/m^2 of rIFN- may be optimal. The two CRs observed occurred at this dosage level. Analysis of the data is continuing. This contract is scheduled to expire on October 31, 1989.

PITTSBURGH, UNIVERSITY OF (N01-CM6-7893)

This is a Phase Ib study to determine the optimal therapeutic and immunomodulatory dose of monoclonal antibody R24 in patients with malignant melanoma. Previous studies have not determined the maximum tolerated dose or optimal dose of the antibody, though they have shown promising therapeutic results clinically. The objectives of the trial design include: (1) to determine pharmacokinetics of the R24 antibody; (2) to perform in situ antibody localization studies; (3) to assay antibody directed cellular cytotoxicity function of peripheral blood mononuclear cells, serum Ig levels, complement levels, natural killer activity peripheral blood leukocyte phenotype, complement-dependent cytotoxicity, phenotypic analysis of tumor infiltrating lymphocytes, binding of monoclonal antibody to Leu-11+ circulating cells, and inflammatory response mediators at the tumor site; and (4) to evaluate antitumor responses. R24 for this study has recently completed production and is undergoing testing.

Another segment of this contract is a Phase Ib trial in melanoma or renal cell cancer, in order to evaluate a new method developed by this group for selecting adherent lymphokine activated killer (A-LAK) from cultures of human peripheral blood mononuclear cells. Isolated cells are enriched for CD3-/Leu 19+ cells which have higher cytolytic activity per cell than is achieved with unselected LAK cell preparations. The clinical usefulness of this approach will be tested using three groups of nine patients with infusions of different dose levelsof A-LAK cells, standardized by activity against unfractionated cultures of LAK cells. A-LAK is harvested from peripheral blood, grown over 10-15 days, and reinfused on day 1.

Interleukin-2 (IL-2) (Cetus) is given at 10^6 U/m^2/day x 5 by continuous infusion. The objectives of the trial design are: (1) evaluation of toxicities and antitumor responses; (2) ^{111}Indium-localization studies in selected patients; and (3) determination of immunological parameters during therapy. Over 37 patients have been studied for A-LAK generation. Approximately 1/3 of patients are able to generate A-LAK cells with sufficient proliferation to allow entry into the trial. Treatment side effects include fever, hypotension, chills, mild nausea, weight gain, and tumor-associated pain. Clinical accrual is still underway. This contract is scheduled to expire on January 29, 1990.

SOUTHERN CALIFORNIA, UNIVERSITY OF (N01-CM4-7675)

This contract supports Phase Ib clinical studies of the monoclonal antibody Lym-1, alone and in combination with interleukin-2 (IL-2), for the treatment of B-cell lymphoma. Objectives of the trial are determination of monoclonal antibody binding, antigen modulation, pharmacokinetics, immunomodulation, and antitumor effects of the antibody and IL-2 combination. Lym-1 is currently in production for this study. This contract is scheduled to expire on January 30, 1990.

T & B BIOCLONE, INC. (N44-CM8-7779) (SMALL BUSINESS INNOVATION RESEARCH Program)

An immunoassay developed during the initial period of this contract was evaluated clinically in the United States and Japan to determine its efficacy as a diagnostic and prognostic indicator of lung cancer. The key element of the assay is the monoclonal antibody, 5E8, which binds a membrane-associated glycoprotein gp160, that is expressed on >70% of the human lung tumors tested. Phase II also focused on the *in vivo* evaluation of immunospecific liposomes for the delivery of AraC. This monoclonal antibody could prove to be a very effective antitumor agent for lung cancer as well as a useful *in vitro* diagnostic tool. This contract expired May 31, 1989.

TEXAS, UNIVERSITY OF (M.D. ANDERSON CANCER CENTER) (N01-CM6-7899)

This contract supports a Phase Ib study of murine monoclonal antibody 14.G2a, against sialogangioside GD3 in combination with interferon-alpha (INF-α) in patients with measurable Stage III malignant melanoma. The objectives of this study are: (1) to determine biodistribution and tumor localization of the radiolabeled monoclonal antibody, alone and in combination with IFN-α; (2) to study the effect of IFN-α on monoclonal antibody pharmacokinetics; (3) to study monoclonal antibody binding to tumor tissues *in vivo*; (4) to determine immunomodulatory effects of the monoclonal antibody alone or in combination with IFN-α; (5) to test for human anti-mouse antibody and tumor infiltrating lymphocytes; and (6) to determine toxicities and antitumor effects. A 14.G2a is in production for this study. This contract is scheduled to expire on January 30, 1990.

TEXAS, UNIVERSITY OF (M.D. ANDERSON CANCER CENTER) (NO1-CM8-7287)

This clinical contract supports the design and conduct of Phase I/II clinical trials of monoclonal antibodies, immunoconjugates and other targeting agents alone or in combination with other investigational or standard therapy. These trials will focus on studies of mechanisms of antitumor response and immune modulation. Current areas of emphasis include the elucidation of complex interactions between the biological agent(s), the host effector cells and the tumor which may lead to tumor regression. Thus, antibody directed cellular cytotoxicity, tumor infiltrating lymphocyte activation, and the induced inflammatory response will be under scrutiny. In addition, detailed studies will be performed of pharmacokinetics and biodistribution, which are intended to lead to better application of monoclonal antibodies to deliver radionuclides, toxins and drugs to tumor cell targets. Of special interest will be the influence on these processes of specific modifications of monoclonal antibodies, such as changes in affinity, isotype, chimerization, and substitution of F(ab')2 fragments. This contract is shared between the Biological Resources Branch and the Cancer Therapy Evaluation Branch in the Division of Cancer Treatment. This a cost reimbursement contract which is to be awarded on May 20, 1989 and will expire on May 19, 1994.

WISCONSIN, UNIVERSITY OF (NO1-CM4-7669)

This contract supports a Phase Ib trial to assess the modulation by interferon-gamma (IFN-) of the Tag-72 antigen, reactive with monoclonal antibody B72.3, on ovarian cancer cells in patients with malignant ascites. Three cohorts of patients will be treated with escalating doses of IFN- (Biogen) ranging from 0.1 MU to 50 MU intraperitoneally weekly over a five-week period, followed by monoclonal antibody first alone and then monoclonal antibody together with the optimal biological doses of IFN- . The objectives are: (1) to study serial specimens of ascites for monocyte numbers, activation markers, and cytotoxicity; (2) to assay monoclonal antibody and IFN pharmacokinetics antigen shedding and induction of anti-IFN, anti-mouse Ig, and anti-idiotype responses. The IFN- antigen modulation trial is underway and results are still preliminary. Early analysis of data suggest a positive effect of IFN on antigen shedding. A second clinical trial to evaluate murine monoclonal antibody + IFN- given systemically to patients with solid tumor, will then be designed based on this experience. This contract is scheduled to expire on January 29, 1990.

Another segment of this contract supports a successor study to a previous Biological Response Modifiers Program contract (NO1-CM4-7669). Using the interleukin-2 (IL-2) (Hoffmann-LaRoche) dosing schedule developed previously: 3 x 10^2 U/m^2/day by continuous infusion for 4 days of each week, repeated for 4 weeks. Patients are now apheresed for peripheral blood mononuclear cells which are then cultured to produce lymphokine activated killer (LAK) cells ex vivo. These cells are re-infused during the next week's IL-2 infusion. Objectives of this study are: (1) to evaluate immunologic and biologic effects, and the toxicities and tolerability of multiple infusions of autologous LAK cells in this regimen; and (2) to determine biologic effects of long-term IL-2 "maintenance" therapy, given for several months after LAK treatment. Eleven patients have been treated.

One/11 PR was seen. There was increased toxicity associated with LAK cells including worsening severity of chills, rigors, hypotension, and complications of indwelling lines (thrombosis, infections). A modified protocol is being designed to attempt to reduce LAK-related toxicity. This contract is scheduled to expire on January 29, 1990.

WISCONSIN, UNIVERSITY OF (NO1-CM8-7290)

This clinical contract supports the design and conduct of Phase I/II clinical studies of cytokines and immunomodulators. These studies focus on elucidation of specific mechanisms involved in the immune modulation and antitumor responses associated with biologic agents, alone or in combination with other modalities. This is a cost reimbursement contract which expires on September 29, 1993.

CANCER THERAPY EVALUATION PROGRAM

BIOTEK, INC. (N43-CM9-7604) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract is to develop heat activated microparticle delivery systems for the delivery of chemotherapeutic agents that could be used in conjunction with therapeutic hyperthermia or hyperthermia plus radiation. The goal during Phase I is to determine the feasibility of preparing the heat activated microparticles using a "model drug" and to evaluate its performance in vitro by measuring the rate of drug release as a function of temperature from 37°C to 42°C. All the microparticles will also be characterized as to drug content, size, distribution and surface morphology. The release of model drug from the most promising formulation will be evaluated in mice.

CALIFORNIA, UNIVERSITY OF (NO1-CM7-3702)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

CITY OF HOPE NATIONAL MEDICAL CENTER (NO1-CM7-3703)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

EMMES CORPORATION (NO1-CM6-7908)

This contract provides support to CTEP in two areas: a) direct organizational data management and statistical support for specific clinical trials (currently The Testicular Cancer Intergroup, Intergroup Pancreas Study, the Head and Neck Follow-up Efforts and extramural LAK/IL-2 trials); and b) information management assistance to Clinical Investigations Branch professional staff in the analysis of methodology and data emanating from the extramural program. A recent area of involvement has been the CTEP initiated treatment protocols for which EMMES has assisted in protocol and forms development and performs operations and data management functions.

INFORMATION MANAGEMENT SERVICES, INC., (NO1-CM6-7810)

This contract supports the maintenance of the computer aspects of the following CTEP information systems: The CTEP-IS, the Phase II System, and the Drug Distribution and Protocol Monitoring System (DDPMS) with further development of linkages between these systems and with outside databases. The CTEP-Information system (CTEP-IS) provides computer capabilities to index, track, select, sort, and locate clinical trials. Currently there are over 9,000 studies in the system, with 275 trials in the CTEP or PDQ review process at any one time. About 1,000 new studies are added each year with some 1,500 amendments to active studies. The Main System Investigator Directory includes 1,300 investigator addresses and/or phone numbers. The Phase II Results Database (PH II) is a subset of 2,000 studies in the Main System which provides scientific results of Phase II single agent clinical trials primarily for the medical staff and for publication purposes. Users can still access the Phase II system, but it has been phased out and is no longer updated.
The Drug Distribution and Protocol Monitoring System (DDPMS) is a computer system and support service which is operated by VSE Corporation, subcontractor to IMS, Inc. The DDPMS is a data base used to verify the accuracy of drug requests and to transmit and record drug shipment information as required by the Food and Drug Administration. The system includes a protocol file, a file of NCI registered investigators (which is updated via an annual reregistration procedure), a drug file and several supportive ancillary files. A new addition is an Electronic Clinical Drug Request system used to expedite and verify requests transmitted electronically from investigators to NCI. In addition to monitoring investigational drug distribution, the system provides computer support service and management information to the program. It contains more than 1,000 active protocol records; 6,600 active investigator records; about 150 drugs and numerous dosage forms; 30,000 drug requests are verified, recorded and processed annually; and, about 550 investigators are registered each month. Numerous special queries and hard copy reports are produced as needed. The system is run using the DCRT mainframe computer.

JOHNS HOPKINS UNIVERSITY (NO1-CM5-7738)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents sponsored by the Division of Cancer Treatment or to conduct Phase I clinical studies with drug combinations or bone marrow transplantation as mutually agreed upon. The contractor has completed Phase I studies with oxantrazole, taxol in leukemia and trimetrexate (abnormal organ function). Trials which include pharmacology studies are being done with gamma interferon in head and neck cancer, 4-ipomenaol, HMBA in myelodysplastic syndrome, and HMBA in oral leukoplasia. Other agents being studied include hepsulfam, taxol and cisplatin, recombinant TNF and etopside, and trimetrexate in patients with renal dysfunction or hepatic dysfunction.

LOYOLA UNIVERSITY (NO1-CM7-3704)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of

human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

MARYLAND, UNIVERSITY OF (NO1-CM5-7734)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents (or combination of agents) sponsored by the Division of Cancer Treatment. The contractor is conducting and/or has completed Phase I studies with menogaril, CBDCA and etopside, IP cisplatin and melphalan, cyclophosphamide and IL-2, uridine and 5-FU, HMBA in myelodysplastic syndromes, IP teroxirone, cisplatin and IL-2, liposome encapsulated doxorubicin and HMBA. Pharmacokinetic studies have been undertaken as part of these trials. Other trials include chloroquinozaline sulfate and doxorubicin, ICRF-187, and GM-CSF. The Phase I contracts are currently being recompeted for award in fiscal year 1990.

MAYO FOUNDATION (N01-CM5-7733)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents (or combination of agents) sponsored by the Division of Cancer Treatment and Phase II/III studies in patients with disseminated solid tumors and leukemia. The contractor is conducting and/or has completed Phase I studies with 6-thioguanine, oxantrazole, and pyrazine diazohydroxide. Dose escalations in the oxantrazole have been based on a pharmacologically guided dose escalation scheme. The contractor is conducting and/or has completed Phase II studies of menogaril in ovarian cancer; ifosfamide/VP-16 for sarcoma; intraperitoneal DHAD for ovarian cancer; TNF for melanoma; DHAC for melanoma; CTX, CBDCA, GM-CSF (pilot),; IFOS + other CT in osteosarcoma; and IV HXM in ovarian cancer. Protocols in development include amonafide in colon cancer; GM-CSF, IFX, VP-16, ADR in pediatric sarcomas; TNF for prostate cancer; adriamycin pharmacokinetics; 5-FU + IFN in advanced cancer; 5-FU, levamisole, leucovorin for colon cancer; and IV 6-thioguanine for unfavorable lymphoma. The Phase I and II/III contracts are currently being recompeted for award in fiscal year 1990.

MEDICAL LASER RESEARCH AND DEVELOPMENT CORPORATION (N44-CM7-7832)
(SMALL BUSINESS INNOVATION RESEARCH Program)

This contract is to develop a surgical laser system which can remove tumors or other tissues with minimal blood loss, precision, and minimal damage to adjacent tissue. The Phase I program was a demonstration of laser control parameters required for hemostatic action with minimal adjacent tissue damage in a murine model *in vivo*. Phase II will include demonstration of laser scalpel action, operator interface design, and system integration, providing a prototype surgical laser system. Demonstration of the efficacy of this system will include acute and long-term (wound healing) studies *in vivo*. Presently available surgical laser systems produce char and hemostasis or no char and no hemostasis. The surgical laser system proposed herein provides hemostasis while making clean sharp cuts with no char formation and minimal adjacent tissue damage. One of the advantages of this system is that the hemostasis laser can be adjusted independently of the cutting laser, thereby providing the

opportunity to optimize each system (cutting and hemostatic) independently. This contract was completed on June 29, 1989.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NO1-CM5-7732)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents (or combination of agents) sponsored by the Division of Cancer Treatment and Phase II/III studies in patients with disseminated solid tumors and leukemia. The contractor is conducting and/or has completed Phase I studies with PALA/MTX/LEU/5FU, HMBA given as a 10 day continuous infusion and merbarone. Pharmacology studies have been undertaken as part of these trials. Other Phase I studies include deoxyspergualin and chronic low dose HMBA in patients with carcinoma in situ of the urinary bladder, and FAMP and chlorambucil in patients with CLL, chloroaquinoxaline sulfonamide, and liposome encapsulated doxorubicin. Phase II/III studies include carboplatin (CBDCA) for osteosarcoma; CBDCA plus VP-16 and bleomycin for advanced poor risk germ cell tumors; isopropylamine CHIP for advanced germ cell tumors; CBDCA for advanced APUD tumors; trimetrexate in endocrine resistant metastatic prostatic carcinoma; menogaril for advanced malignant mesothelioma; gallium nitrate for measurable urothelial tract cancer; PALA, MTX and 5-FU-leucovorin for adenocarcinoma of unknown origin; HMBA for malignant melanoma; HMBA for myelodyplastic syndrome; amonafide for non-small cell lung cancer; high dose MTX for high grade sarcoma of bone or cartilage, excluding osteogenic sarcoma; carboplatin/velban for adenocarcinoma of the upper GI tract; didemnin B for advanced renal cell carcinoma; a randomized study of etopside and cisplatin and etopside and carboplatin for good risk patients with advanced germ cell tumors; and HD-MVAC with leucovorin rescue for measurable urothelial tract cancer. The Phase I and II/III contracts are currently being recompeted for award in fiscal year 1990.

MONTEFIORE MEDICAL CENTER (NO1-CM7-3705)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

NEW ENGLAND MEDICAL CENTER HOSPITALS, INC. (NO1-CM7-3706)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to

patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

OHIO STATE UNIVERSITY RESEARCH FOUNDATION (NO1-CM5-7736)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents sponsored by the Division of Cancer Treatment or to conduct Phase I clinical studies with drug combinations or bone marrow transplantation as mutually agreed upon. The Phase I trials of merbarone and fazarabine are nearing completion. Other Phase I studies include deoxycoformycin (dCF) in patients with renal dysfunction; a Phase I trial of the combination of dCF and fludarabine phosphate; FAA; fostriecin in patients with acute leukemia; and ipomeanol in patients with lung carcinoma. Other agents being studied include BSO and melphalan, pyrazine diazohydroxide and oral fludarabine. Extensive pharmacokinetic studies have been conducted. The Phase I contracts are currently being recompeted for award in fiscal year 1990.

OHIO STATE UNIVERSITY RESEARCH FOUNDATION (NO1-CM7-3701)

The principal objective of this project is the pharmacokinetic analysis of samples from patients with malignant disease accrued to studies using either single or combinations of a new and/or established anticancer agent(s). The studies are primarily concerned with the measurement of the agent and/or metabolite levels in plasma, tissue, urine and feces in order to determine the distribution, metabolism and elimination, as well as target effect(s) or absorption if the agent is administered orally or by some route other than i.v. The results of these studies are analyzed in particular to help establish the most effective dosage schedule and to anticipate any situation where altered organ functions could lead to a different pattern of drug distribution, metabolism and elimination resulting in toxicities which would not be expected otherwise. Two agents are evaluated annually.

SOCIAL & SCIENTIFIC SYSTEMS, INC. (NO1-CM2-5606)

This contractor provides support services for conference management and associated general logistical activities for the Cancer Therapy Evaluation Program. Logistics support includes various technical and clerical tasks ranging from report design and preparation to routine typing. Conference support includes both pre- and post-conference management activities necessary to successfully conduct large, as well as small, meetings and provide the results thereof to the biomedical research community.

SOCIAL & SCIENTIFIC SYSTEMS, INC. (NO1-CM7-3709)

The contract provides support services for the operations of the Cancer Therapy Evaluation Program, particularly the Investigational Drug Branch, Regulatory Affairs Branch, Biometric Research Branch and the Protocol and Information Office. The contractor is responsible for the data collection/ compilation, technical report preparation, administrative coordination, and general logistical support, particularly in the area of investigational drugs which are subject to regulation by the Food and Drug Administration (FDA).

The contract is divided into two principal parts: 1) clinical research/FDA compliance support which includes clinical research support, regulatory affairs support and drug distribution support; and 2) protocol and information support which includes protocol review and approval, tracking and coordination, protocol information tracking and dissemination, and protocol results and publications.

SOUTHERN RESEARCH INSTITUTE (NO1-CM6-7724)

This contract provided a resource to evaluate the antitumor activity and toxicity of antitumor agents using murine leukemia and solid tumor models. The emphasis was on the evaluation of antitumor synergy between agents with activity in clinical trials. Pairs of agents with demonstrated antitumor activity were evaluated for the development of combination regimens for clinical trials. Studies fell into one of the following categories: combinations of cytotoxic agents; combinations of biologic agents; combinations of biologic and cytotoxic agents; single agent antitumor activity of selected biologic agents; and evaluation of biochemical modulation in vivo. The results of these preclinical studies and clinical trials were compared to determine the utility of this approach in designing combination regimens for clinical trial. This contract expired on February 15, 1989.

TEXAS, UNIVERSITY OF, HEALTH SCIENCE CENTER (NO1-CM5-7737)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents sponsored by the Division of Cancer Treatment or to conduct Phase I clinical studies with drug combinations or bone marrow transplantation as mutually agreed upon. The contractor is conducting and/or has completed Phase I studies with deoxyspergualin, taxol and trimetrexate (leukemias). Other studies include continious intravenous infusion of TNF; hepsulfam; and liposome encapsulated doxorubicin. Pharmacokinetic studies have been conducted on approximately 50% of the patients accrued. The Phase I contracts are currently being recompeted for award in fiscal year 1990.

TEXAS, UNIVERSITY OF, HEALTH SCIENCE CENTER, SAN ANTONIO (NO1-CM7-3707)

This contract is one of a group of contractors which enter patients onto common protocols. Each contractor performs clinical evaluations of treatment regimens utilizing activated leukocytes sponsored by the Division of Cancer Treatment under an investigational new drug application (IND). The objectives of these protocols are to: 1) determine the antitumor activity of activating agents in combination with activated leukocytes in a variety of human cancers in patients with advanced disease; 2) define the toxicities of activating agents administered in combination with activated leukocytes to patients with advanced cancer; and 3) evaluate new methods for activation of leukocytes and determine the antitumor activity of activated leukocytes generated using the new methodology.

TEXAS, UNIVERSITY OF, SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (NO1-CM5-7739)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents (or combination of agents) sponsored by the Division of Cancer Treatment and Phase II/III studies in patients with disseminated solid tumors and leukemia. The contractor is conducting and/or has completed Phase I studies with fostriecin and FAA given by prolonged continuous infusion, FAA and IUDR given by continuous hepatic artery infusion, MoAb xomazyme-H65 in patients with CLL and NHL, and studies of cyclophosphamide, TIL and alpha-interferon in patients with melanoma and GU malignancies. Phase II studies include: intravenous carboplatin for malignant primary brain tumors; dihydro-5-azacytidine in malignant mesothelioma; trimetrexate for metastatic bladder cancer; trimetrexate for metastatic or unresectable primary advanced carcinoma of the uterine cervix; taxol for metastatic melanoma; methyl-CCNU in combination with 5-FU plus folinic acid for metastatic colorectal carcinoma advanced colorectal carcinoma and advanced colorectal cancer; didemnin B in non-small cell lung cancer, advanced, measurable adenocarcinoma of the pancreas and, metastatic breast cancer; fludarabine plus prednisone in advanced chronic lymphocytic leukemia; high dose melphalan and total body irradiation with autologous bone marrow rescue as consolidation therapy for multiple myeloma in second remission following VAD or dexamethasone; carboplatin plus cisplatin in recurrent or advanced squamous cell carcinoma of the head and neck; intravenous melphalan for advanced, measurable colorectal cancer; pilot study of carboplatin and continuous infusion bleomycin in advanced or recurrent squamous cell carcinoma of the head and neck; I.V. menogaril in primary malignant brain tumors; I.V. 6-thioguanine for metastatic or locally advanced pancreatic adenocarcinoma or metastatic or locally advanced gastric adenocarcinoma; and 6-thioguanine or trimetrexate for patients with metastatic or inoperable non-small cell lung cancer. The contractor is also conducting a Phase III study of newly diagnosed adult acute myelogenous and undifferentiated leukemia. The Phase I/II/III clinical trials contracts are being recompeted for new awards in Fiscal Year 1990.

THERADEX SYSTEMS, INC. (NO1-CM8-7208)

The objective of this contract is to provide a clinical trials monitoring service for the Phase I/II CTEP and BRMP investigators and all other investigators using NCI-sponsored investigational drugs. This service has four components: 1) a central data management resource for investigators conducting Phase I clinical trials, Phase II/LAK-IL2 trials and designated high priority Phase II trials; 2) an on-site audit resource for DCT to assure that Phase I/II contractors are in compliance with federal regulations; 3) co-site visiting cooperative group members as observers of peer audits; and 4) on-site auditing of all other individual investigators conducting investigational trials. This contract was awarded on June 30, 1988 for a five year period.

WISCONSIN, UNIVERSITY OF (NO1-CM5-7735)

This contract is designed to conduct Phase I and clinical pharmacokinetic studies with new anticancer agents sponsored by the Division of Cancer Treatment or to conduct Phase I clinical studies with drug combinations or bone marrow transplantation as mutually agreed upon. The contractor is conducting and/or has completed Phase I studies with TNF, SR-2508/CTX fazarabine, and the

combination of TNF and gamma interferon. Pharmacology studies or monitoring of biological endpoints have been undertaken in conjuction with these studies. Other Phase I studies being undertaken include L-Buthionine sulfoximine and melphalan, whole body hyperthermia and carboplastin, and 5-fluorouracil leucovorin and dipyrimidamole. The Phase I contracts are currently being recompeted for award in fiscal year 1990.

CLINICAL ONCOLOGY PROGRAM

BIONETICS RESEARCH, INC. (N01-CM8-7236)

This contract supports Surgery Branch research by providing human lymphokine activated killer cells and tumor infiltrating lymphocytes for therapeutic trials. This research is directed toward developing new adoptive immunotherapies for the treatment of cancer.

DEPARTMENT OF ENERGY (ARGONNE NATIONAL LABORATORIES) (Y01-CM7-0148)

This contract supports the Radioimmune Chemistry Section, Radiation Oncology Branch, by providing generators from which monoclonal antibody, radiolabeling agents are derived.

GEORGETOWN UNIVERSITY, DEPARTMENT OF RADIATION MEDICINE (N01-CM9-0001)

This contract supports the Radiation Oncology Branch by providing radiation therapy technology support for the Radiation Oncology Branch Clinic; this includes technology, dosimetry, physics, medical and nursing support.

HAZELTON LABORATORIES AMERICA, INC. (N01-CM4-7649)

This contact supports the research efforts of the Surgery Branch through the maintenance and transfer of tumor bearing laboratory animal models.

LIBRARY OF CONGRESS (Y01-CM5-0133)

The Library of Congress will provide administrative services to the National Cancer Institute, Medicine Branch, to facilitate procurement, installation and training of library support and information retrieval services and associated technical services under the FEDLINK bylaws.

MEDICAL ILLNESS COUNSELING CENTER (N01-CM8-7263)

This contract provides the Clinical Oncology Program with neuropsychological testing for children and adults with HIV infection for the purpose of monitoring neuroencephalopathy and the benefits of therapeutic interventions. This research will provide careful assessment of encephalopathy changes in relation to therapeutic modalities for HIV infection.

NAVAL HOSPITAL, BETHESDA REGION (Y01-CM8-0159)

The overall objectives and specific accomplishments of the Interagency Agreement between the Naval Hospital, Bethesda (NH-BETH), and the National Cancer Institute (NCI) that we hope to achieve are: (1) Performance of clinical investigations into the diagnosis, staging, and treatment of a variety of malignant diseases through the mechanism of IRB approved clinical protocols. These protocols include studies developed by the NCI-Navy and Naval Hospital, Bethesda, Hematology-Oncology Branches, and the NCI Clinical Center Branches. They represent a collaborative effort between the NH-BETH and the NCI; (2) Integration of the NCI into the patient treatment program of the NH-BETH; and (3) Development of a laboratory program by NCI-Navy Medical Oncology Branch to investigate the biology of human tumors.

ORKAND CORPORATION (NO1-CM6-7716)

This contract supports the Clinical Oncology Program with computer programming expertise for the development of clinical information systems and with data technician services for the maintenance and utilization of these systems. A wide variety of systems have been developed and are maintained for the Clinical Branches of the Clinical Oncology Program.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (YO1-CM5-0134)

The USUHS will provide one faculty position to support the collaborative efforts for research and educational opportunities between the USUHS, National Cancer Institute (NCI), and Naval Hospital, Bethesda, Maryland (NH-BETH). These collaborative efforts are mutually beneficial to both agencies due to the furthering of both research interests and educational opportunities.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (YO1-CM6-0141)

The purpose of this agreement is to document collaborative efforts between the USUHS, National Cancer Institute (NCI), and Naval Hospital, Bethesda, Maryland (NH-BETH). The USUHS will provide positions to support the collaborative efforts for research and educational opportunities between the two agencies.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (YO1-CM7-5050)

The purpose of this Memorandum of Understanding (MOU) is to permit collaborative efforts among the USUHS, National Cancer Institute (NCI), and the Naval Hospital, Bethesda, Maryland (NH-BETH) to improve quality of care of patients with cancer and related diseases at NH-BETH. It is recognized by all parties that quality of radiation therapy services is a critical determinant of the quality of a clinical oncology program. All parties recognize that the quality of radiation therapy services are necessary to the research mission of the NCI-NH-BETH medical oncology program and to the undergraduate education mission of USUHS.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (YO1-CM8-0158)

The purpose of this agreement is to document collaborative efforts between the USUHS, National Cancer Institute (NCI), and Naval Hospital, Bethesda, Maryland (NH-BETH). These efforts are mutually beneficial to both Institutes due to the furthering of research interests and educational opportunities. The USUHS will provide positions within the Department of Pharmacology. These positions shall be used to employ staff who will work on specific research projects identified by the USUHS and NCI and be responsible for specific duties related to a USUHS faculty appointee.

DEVELOPMENTAL THERAPEUTICS PROGRAM

ABBOTT LABORATORIES (NO1-CM6-7862)

This resource contract provides the Division of Cancer Treatment with facilities and personnel for the development and production of oral dosage forms of investigational anti-AIDS drugs. The dosage forms are manufactured in conformity with U.S. FDS Current Good Manufacturing Practices and in a manner to protect personnel from exposure to potentially toxic agents. The contractor is also responsible for all required quality control tests on each agent prepared. These dosage forms are packaged, labeled, and shipped to the National Cancer Institute for subsequent redistribution to clinical investigators. This contract is being recompeted.

AERON BIOTECHNOLOGY, INC. (N44-CM7-7826) (SMALL BUSINESS INNOVATION RESEARCH Program)

The goal of this project is to develop an in vitro assay for predicting sensitivity to methotrexate in human breast cancer specimens. Two uptake assays are being optimized and compared: accumulation of fluoresceinated-methotrexate [F-MTX] as measured with a fluoroescence activated cell sorter (FACS) and uptake of tritiated-methotrexate [^{3}H-MTX] using conventional liquid scintillation counting techniques. Assay conditions have been developed using human breast cancer cell lines. Validation studies with tumor specimens are currently in progress. The assays may be useful in determining patient sensitivity to methotrexate and in evaluating mechanisms of drug resistance. The contract will expire in September 1989.

AEROJET STRATEGIC PROPULSION COMPANY (NO1-CM8-7273)

This service preparative contract provides for the resynthesis of a variety of compounds required for clinical evaluation in Phase II & III trials as potential anti-AIDS agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

ALABAMA, UNIVERSITY OF (NO1-CM6-7971)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

ALABAMA, UNIVERSITY OF (N01-CM8-7267)

This contract is for chemical synthesis and drug design of a variety of compounds for evaluation as potential anti-AIDS agents. The specific objectives of this project are: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that possess elements of both congener and prodrug; (c) to synthesize compounds related to products of natural origin and other related heterocycles; and (d) to synthesize anti-sense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

ALDRICH CHEMICAL COMPANY, INC. (N01-CM6-7771)

This service preparative contract provides for the resynthesis of a variety of compounds required for toxicology and clinical evaluation in Phase I trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

ALDRICH CHEMICAL COMPANY, INC. (N01-CM6-7929)

This service preparative contract provides for the resynthesis of a variety of compounds required for clinical evaluation in Phase II and III trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

ARTHUR D. LITTLE, INC. (N01-CM8-7284)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the contractor through a Task Assignment mechanism. These studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) in vitro stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following i.v. bolus and infusion dosing in mice, rats, and dogs; (4) identification of drug metabolites. Data obtained in these investigations is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of drug in biological fluids. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Pre-clinical pharmacology studies of potential anti-AIDS agents are generally performed in parallel with, and are designed to aid in the interpretation of preclinical toxicology evaluations. Together, these investigations provide required data for INDA filing as well as a rational basis for the clinical Phase I starting dose and dose escalation scheme.

ASH STEVENS, INC. (N01-CM6-7822)

This service preparative contract provides for the resynthesis of a variety of compounds required for clinical evaluation in Phase I trials as potential anti-AIDS agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

ASH STEVENS, INC. (N01-CM6-7871)

This service preparative contract provides for the resynthesis of a variety of compounds required for the clinical evaluation in Phase II and III trials as potential anti-AIDS agents. The compounds prepard are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

ASH STEVENS, INC. (N01-CM6-7927)

This service preparative contract provides for the resynthesis of a variety of compounds srequired for the clinical evaluation in Phase II and III trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

BATTELLE MEMORIAL INSTITUTE (N01-CM6-7869)

This contract is for the performance of preclinical toxicology studies of potential therapeutic agents for treating AIDS. These preclinical studies primarily are designed to relate target organ toxicity to plasma drug concentration and in vitro efficacy. Protocols include pharmacokinetic and bioavailability studies in rodents and dogs, cerebrospinal fluid penetration by the drug in dogs, continuous intravenous infusion toxicity studies in dogs and 28 day multiple dose toxicity studies in dogs and rodents. The data from these studies are used to prepare attachment 6a for the INDA. This is the third year of a three year contract.

BATTELLE MEMORIAL INSTITUTE (N01-CM9-7612)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited
number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors.

BATTELLE MEMORIAL INSTITUTE (NO1-CM9-7617)

This contract is for the performance of preclinical toxicology studies of potential therapeutic agents for treating AIDS. These preclinical studies primarily are designed to relate target organ toxicity to plasma drug concentration and in vitro efficacy. Protocols include pharmacokinetic and bioavailability studies in rodents and dogs, cerebrospinal fluid penetration by the drug in dogs, continuous intravenous infusion toxicity studies in dogs and 28 day multiple dose toxicity studies in dogs and rodents. The data from these studies are used to prepare attachment 6a for the INDA. This is the first year of a three year contract.

BEN VENUE LABORATORIES, INC. (NO1-CM6-7865)

This contractor is required to manufacture dosage forms of compounds with potential activity in AIDS. Compounds are selected and provided by the Government. The contractor is responsible for manufacturing these dosage forms in conformity to U.S. FDA Current Good Manufacturing Practice regulations. Other responsibilities include quality control testing, packaging, labelling and distribution of the final product to the National Cancer Institute.

BEN VENUE LABORATORIES, INC. (NO1-CM7-3719)

This resource contract provides for the development and production of parenteral clinical dosage forms of anticancer agents. The contractor has the capability of preparing production batches of liquid-filled and lyophilized sterile products. Specifically, the contractor performs the following services: (1) formulation development of parenteral products; (2) production of sterile products; and (3) quality assurance testing of finished products. All products are packaged, labeled, and shipped to the National Cancer Institute for subsequent redistribution to clinical investigators.

CAPITOL TECHNOLOGY INFORMATION SERVICES (NO1-CM9-7600)

This service contract provides for the installation, maintenance and operation of a anti-AIDS relational database integrated with the heirarchial chemistry-based database. The integrated system will permit queries of the relational database to interface with the heirarchial chemistry database and queries of the chemical heirarchial database to interface with the relational biological database.

CHARLES RIVER LABORATORIES (NO1-CM9-7575) (Formerly NO1-CM6-7881)

This contract is a Primary Genetic Center. Rederived associated flora pedigreed starts are supplied to this contract for propagation into isolator maintained foundation colonies. These foundation colonies supply breeders on a weekly basis to pedigreed expansion colonies which support the production colonies. Both the pedigreed expansion and production colonies are maintained in a barrier room environment. Offspring from the production colonies are used for hybrid production and the many NCI research activities.

CIVILIZED SOFTWARE (N44-CM7-7811) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase II project has as its target product a PC version of the mathematical modelling and curve fitting program MLAB. MLAB currently is written in SAIL and runs on DCRT's DEC10. The new program is to be written in C and is targeted for the IBM AT class PC. This project terminates on Sept. 29, 1989.

CONNECTICUT, UNIVERSITY OF (NO1-CM5-7692)

This contract furnished extracts and cryopreserved cultures from 500 strains of unique genera of fungi per year for antitumor and anti-AIDS evaluations. The contract called for fermentation in three different media, harvest at two stages of the growth cycle and preparation of two extracts, a mycelial extract and a lyophilized broth extract. More than 1100 unique organisms were cultivated, preserved and extracted. The contract was given a no cost extension to December, 1988 to complete packaging, data entry and delivery. The contract was completed with delivery of 6600 extracts. Testing of those extracts in the anti-AIDS screen has already identified 8 organisms which are producing strong in vitro activity, and scale-up of fermentation of those organisms has begun in order to provide material for chemical investigation at the National Cancer Institute.

CREARE (N44-CM8-7966) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase II project, like the Phase II project awarded to Civilized Software, has as its target product a PC version of the mathematical modelling program MLAB. Also to be written in C, this version has as its target computer a 80386 microprocessor based 32-bit machine running under the Xenix operating system.

DARTMOUTH COLLEGE (NO1-CM6-7976)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

ERC INTERNATIONAL (NO1-CM6-7920)

This resource contract provides the Division of Cancer Treatment with storage and distribution capabilities for the large volume of investigational drugs used in clinical trials. Approved orders for clinical drugs are packaged and shipped to destinations around the world. The contractor also provides a computerized inventory management system. This assures the proper rotation of stock, an adequate lead time to obtain new supplies of drugs, and the prompt removal of expired materials. Further, computerized records are kept for all shipments made and returns received, which aids in accountability.

ERCI FACILITIES SERVICE CORPORATION (N01-CM7-3721)

This contractor furnishes the National Cancer Institute with facilities and services for the storage and distribution of synthetic chemicals, bulk chemical drugs and crystalline natural products. Samples are weighed, packaged and shipped to contract screening laboratories and also to various domestic and foreign research institutions. The contract provides for the maintenance of accurate computerized inventory shipping and distribution records. This is an on-going operation and supports all the DTP programs.

FEIN-MARQUART ASSOCIATES (N01-CM8-7276)

This service contract provides for the maintenance of the Drug Information System (DIS) used by the DTP staff, DTP contractors and DTP suppliers. The DIS is a computer-based system, containing information on the nearly half million chemicals that have been tested by the DTP. The information includes graphical chemical structures, sources, physical data, shipping histories, in vivo test results, etc. The system generates off line reports, is accessible online for querying, and is used for monitoring as well. The contractor maintains the files, takes care of software problems as they occur, and responds to DTP requirements for alterations and improvements of the computer programs.

FEIN-MARQUART ASSOCIATES (N01-CM9-7608)

This service contract provides support for the installation of a computerized heirarchical chemistry-oriented database system on a VAX 8820. Although the old 36-bit code had previously been converted to a 32-bit word size, a number of steps are required to complete the changeover.

GEORGIA, UNIVERSITY OF (N01-CM7-3712)

This contractor has the responsibility of performing shelf life evaluation of clinical drugs. The contractor monitors the stability of dosage forms at several storage temperatures. The testing involves the use of multiple analytical methods. The method most frequently used for assay of the stability samples is high performance liquid chromatography (HPLC). The data that is developed is used to verify the stability of NCI's investigational drugs during the clinical trials, and is supplied to the U.S. Food and Drug Administration in support of NCI's IND filings. The contractor also has the responsibility of conducting reserve sample inspections as required by the FDA Current Good Manufacturing Practices.

GEORGIA TECH RESEARCH CORPORATION (N01-CM8-7269)

This contract provides for chemical synthesis and drug design of a variety of compounds for evaluation as potential anti-AIDS agents. The specific objectives of this project are: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that possess elements of both congener and prodrug; (c) to synthesize compounds related to products of natural origin and other related heterocycles; and (d) to synthesize anti-sense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

HARBOR BRANCH OCEANOGRAPHIC INSTITUTE (NO1-CM9-7578)

This interim contract was installed for 14 months to provide continuity in the collection of marine organisms while a new RFP could be issued for the collection of shallow water marine organisms. Since it began in September, 1988, the contractor has delivered 895 samples to the NCI repository and has collected over 600 more that will be delivered later this summer. The organisms are selected to represent the greatest taxonomic and ecological diversity from a broad region of the Indo-Pacific geographical area. Representatives from the phyla Porifera, Chordata, Echinodermata Cnidaria, Annelida and Mollusca have been collected in sufficient quantity to provide adequate sample for full chemistry characterization. Extracts are being evaluated in both the antitumor and anti-AIDS screening assays. This interim contract will be completed in October, 1989.

HARLAN/SPRAGUE-DAWLEY, INC. (NO1-CM2-3911)

This contract operates the Animal Production Area at the Frederick Cancer Research Facility (FCRF). The contract operates as a Primary Genetic Center, Rederivation Center, and Embryo Freezing Center. Strains are received from the NIH Repository for use at the FCRF and distribution to other NCI contract activities. The bulk of the production on this contract is for supplying the animal needs of the researchers located at the FCRF. Animals are also sent from FCRF to other NCI funded research activities. This contract was recompeted and Harlan/Sprague Dawley was awarded a seven year extension of this same contract.

HARLAN/SPRAGUE-DAWLEY, INC. (NO1-CM9-7623) (Formerly N01-CM6-7918)

This contract is a Primary Genetic Center. Rederived associated flora pedigreed starts are supplied to this contract for propagation into isolator maintained foundation colonies. These foundation colonies supply breeders on a weekly basis to pedigreed expansion colonies which support the production colonies. Both the pedigreed expansion and production colonies are maintained in a barrier room environment. Offspring from the production colonies are used for the many NCI research activities.

HAUSER CHEMICAL RESEARCH (NO1-CM8-7219)

This Master Agreement for Master Agreement Orders in the large-scale isolation of anti-AIDS agents from natural sources was established to permit the isolation and purification of active materials from organisms, such as plants and marine invertebrates, for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. As yet, no project has been assigned to the contractor. Responsibility for supervision of this contract was recently transferred from the Natural Products Branch to the Pharmaceutical Resources Branch.

HAUSER CHEMICAL RESEARCH (NO1-CM9-7554) (Formerly NO1-CM8-7221)

This Master Agreement for Master Agreement Orders in the large-scale isolation of anti-tumor agents from natural sources was established to permit the isolation and purification of active materials from organisms, such as plants and marine invertebrates, for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. The Contractor has completed tasks involving the extraction of Bugula neritina and Taxus brevifolia prior to the isolation of Bryostatin (NSC 339555) and Taxol (NSC 125973) respectively, and is currently working on a task to isolate Taxol from the crude extract. Responsibility for supervision of this contract was recently transferred from the Natural Products Branch to the Pharmaceutical Resources Branch.

HAWAII, UNIVERSITY OF (NO1-CM6-7745)

This contract furnishes extracts and preserved cultures from 180 unique strains of blue-green algae (cyanobacteria) per year for antitumor and anti-AIDS evaluations. During the first 2½ years the contractor has delivered 1074 extracts from 537 organisms, as well as 504 cryopreserved, viable cultures. Testing of the extracts in the anti-AIDS screen has identified a series of novel sulfolipids that have shown good activity in the in vitro assay. Emphasis has been placed on taxonomic diversity with a broad distribution over the Orders Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales and Stigonemateles. This 5 year contract was initiated on September 1, 1986.

HAWAII, UNIVERSITY OF (NO1-CM8-7282)

This Master Agreement for Master Agreement Orders for the collection of terrestrial plants for anti-AIDS drugs was established to allow the collection of sufficient material to permit the isolation of active compounds from the plants for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. As yet, no project has been assigned to the contractor.

HAZLETON LABORATORIES AMERICA (NO1-CM6-7931)

This contract is for the performance of preclinical toxicology studies of potential therapeutic agents for treating AIDS. These preclinical studies primarily are designed to relate target organ toxicity to plasma drug concentration and in vitro efficacy. Protocols include pharmacokinetic and bioavailability studies in rodents and dogs, cerebrospinal fluid penetration by the drug in dogs, continuous intravenous infusion toxicity studies in dogs and 28 day multiple dose toxicity studies in dogs and rodents. The data from these studies are used to prepare attachment 6a for the INDA. This is the third year of a three year contract.

H.G. PARS PHARMACEUTICAL LABS., INC. (N01-CM6-7972)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

HIPPLE CANCER RESEARCH CORPORATION (N44-CM7-7829) (SMALL BUSINESS INNOVATION RESEARCH Program)

This is the second and final year of Phase II SBIR contract for the development of a method and instrumentation for the growth of human tumor cell colonies in glass microcapillaries. Several improvements to the instrumentation initially used have been made. Growth kinetics, drug stability in vitro, as well as correlation studies of human tumor in vitro response to specific drugs with that of the patient being tested are being conducted.

HIPPLE CANCER RESEARCH CORPORATION (N44-CM7-7831) SMALL BUSINESS INNOVATION RESEARCH Program)

The objectives of this contract are to standardize in vitro human and murine bone marrow progenitor assays for use in quantitative and qualitative measurements of the toxic effects of known and developing anticancer drugs. The toxicities found in bone marrow culture will be correlated with clinical hematotoxicity. In vitro studies of drug induced myelotoxicity have largely used mouse bone marrow. However, because of the differences in murine and human pharmacokinetics, drug metabolism and drug sensitivity of hematopoietic precursor cells, such murine studies often have not provided useful quantitative data on levels of clinical hematotoxicity in humans. Comparative murine versus human myelotoxicities have enhanced dosing and scheduling in the clinical protocols for both Hepsulfam and L-BSO/L-PAM. The commercialization intent of the SBIR program has been met as the contractor has now made available these stem cell assays to the basic scientific and drug development communities. This SBIR contract terminated on June 29, 1989.

ILLINOIS, UNIVERSITY OF (N01-CM6-7705)

The objective of this contract is to perform a survey of the literature published worldwide on natural products and related fields, and to provide the Project Officer pertinent information required for the evaluation and acquisition of new and novel compounds that may have biological activity useful in the treatment of cancer. Report of biological activity of extracts of plants, animals, bacteria, fungi, and marine organisms are also provided. This contract, awarded for a three-year period, expired on December 30, 1988.

ILLINOIS, UNIVERSITY OF (N01-CM6-7925)

This contractor is undertaking plant collections in South East Asia. The objective is to collect 1,500 plant samples per year for submission to NCI for extraction and screening against a panel of human cancer cell lines and for anti-HIV activity. Collections are currently ongoing in the Philippines, Malaysia, Indonesia, and Papua New Guinea. A number of medicinal plants have been collected in Nepal. Over 4,500 samples have been submitted to the Natural Products Repository at the Frederick Cancer Research Facility in Frederick, MD.

ILLINOIS, UNIVERSITY OF (N01-CM8-7226)

The function of this contract is to conduct a surveillance of current literature published worldwide on natural products and related fields, and to provide the Project Officer with pertinent information needed for the evaluation and acquisition of new and novel compounds that may have biological activity useful in the treatment of AIDS. Biological activity of extracts of plants, animals, bacteria, fungi, and marine organisms are also reported. In addition, the contract provides for retrospective searches of families and genera which show activity of interest in the screening program. This contract has been awarded for a five-year-period ending June 29, 1993.

INTEGRATED GENETICS, INC. (N44-CM8-3717) (SMALL BUSINESS INNOVATION RESEARCH Program)

The objective of this contract is to develop a panel of well-characterized human tumor sublines that express the MDR (multidrug resistance) phenotype for use in large-scale, disease-oriented anticancer drug screening programs. The sublines are obtained either by selection in drug-containing media or by gene transfer techniques. The contractor plans to characterize the sublines for multidrug resistance, mdr gene copy number, growth characteristics, stability, etc. These sublines should provide a valuable resource for the study of molecular mechanisms contributing to the MDR phenotype and should be useful for identifying new anticancer agents for the treatment of drug-resistant neoplasms.

IOWA, UNIVERSITY OF (N01-CM4-7594)

This resource contract provides the Division of Cancer Treatment with facilities and personnel for development and production of oral dosage forms of investigational anticancer drugs. The dosage forms are manufactured in conformity with U.S. Current Good Manufacturing Practices and in a manner to protect personnel from exposure to potentially toxic agents. The contractor is also responsible for all required quality control tests on each product prepared. These dosage forms are packaged, labeled, and shipped to the National Cancer Institute for subsequent redistribution to clinical investigators.

IOWA, UNIVERSITY OF (N01-CM6-7916)

This contract provides services involving dosage form development and manufacture of investigational drugs for subsequent clinical evaluation. Compounds to be formulated are selected and provided by the National Cancer

Institute. The contractor has primarily developed and produced sterile freeze-dried injectable products under this contract. However, the contractor has the capability to produce a wide variety of pharmaceutical dosage forms. The contractor is also responsible for completing all required quality control tests on each lot of drug. All products are packaged, labeled, and shipped to the National Cancer Institute for redistribution to clinical investigators.

IOWA, UNIVERSITY OF (N01-CM6-7979)

This resource contract provides the Division of Cancer Treatment with facilities and personnel for the development and production of oral dosage forms of investigational anti-AIDS drugs. The dosage forms are manufactured in conformity with U.S. FDA Current Good Manufacturing Practices and in a manner to protect personnel from exposure to potentially toxic agents. The contractor is also responsible for all required quality control tests on each agent prepared. These dosage forms are packaged, labeled, and shipped to the National Cancer Institute for subsequent redistribution to clinical investigators.

KANSAS, UNIVERSITY OF (N01-CM6-7912)

This contract investigates approaches to resolve difficult dosage form development problems not amenable to usual solubilization of stabilization methods. The contractor has considerable expertise in the application of molecular complexes and reversible derivatives to improve solubility. The contractor also is responsible for pilot scale preparation and chemical analysis of the formulations developed under this contract. This contract is being recompeted.

MAYO FOUNDATION (N01-CM6-7904)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of Phase I trials of new agents. Task Orders are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on *in vitro* screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was successfully completed and terminated on April 30, 1989.

MAYO FOUNDATION (NO1-CM9-7618)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of Phase I trials of new agents. Task Assignments are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was successfully completed and terminated on April 30, 1989.

METAFLUOR, INC. (NO1-CM9-7614)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors.

MIAMI, UNIVERSITY OF (NO1-CM6-7877)

This contract provides for a complete pathological, parasitical, and microbiological work-up of breeding stock primarily sent from barrier room expansion colonies and the nude mouse production colonies. Special testing is provided on a priority basis when a suspected animal disease problem occurs at an animal supplier facility or a contract research laboratory. All testing is scheduled by the Project Offcier.

MIDWEST RESEARCH INSTITUTE (NO1-CM6-7932)

This contract is for the performance of preclinical toxicology studies of potential therapeutic agents for treating AIDS. These preclinical studies primarily are designed to relate target organ toxicity to plasma drug concentration and in vitro efficacy. Protocols include pharmacokinetic and bioavailability studies in rodents and dogs, cerebrospinal fluid penetration by the drug in dogs, continuous intravenous infusion toxicity studies in dogs and

28 day multiple dose toxicity studies in dogs and rodents. The data from these studies are used to prepare attachment 6a for the INDA. This is the third year of a three year contract.

MIDWEST RESEARCH INSTITUTE (NO1-CM7-3713)

This is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phass of the DTP as potential antitumor agents. The contractor determines identity and purity of the compounds by appropriate methods. The contractor also determines solubility, stability and other physical-chemical properties to aid in formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring.

MIDWEST RESEARCH INSTITUTE (NO1-CM8-7202)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 120 hours; and pharmacokinetics. The data from these studies are used to prepare attachment 6a for the INDA. This is the second year of a five year contract.

MIDWEST RESEARCH INSTITUTE (NO1-CM8-7228)

This is one of two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the DTP as potential anti-AIDS agents. The contractor determines identity and purity of the compounds by appropriate methods. The contractor also determines solubility, stability and other physical-chemical properties to aid in formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring.

MISSOURI, UNIVERSITY OF (NO1-CM6-7723)

This contract provides for a complete pathological, parasitical, and microbiological work-up of breeding stock primarily sent from barrier room expansion colonies and the nude mouse production colonies. Special testing is provided on a priority basis when a suspected animal disease problem occurs at an animal supplier facility in a contract research facility. All testing is scheduled by the Project Officer.

MISSOURI BOTANICAL GARDEN (N01-CM6-7923)

This contractor is undertaking plant collections in Madagascar and adjacent islands, and tropical and subtropical areas of Africa. The objective is to collect 1,500 plant samples per year for submission to NCI for extraction and screening against a panel of human cancer cell lines and for anti-HIV activity. Collections are currently ongoing in the Central African Republic, Gabon and Tanzania, and over 4,000 samples have been submitted to the Natural Products Repository at the Frederick Cancer Research Facility in Frederick, MD.

MORAVEK BIOCHEMICALS, INC. (N44-CM7-7830) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract provides for the radiolabeled synthesis of anticancer compounds for use in preclinical studies. These compounds are unobtainable from commercial sources. All materials are analyzed for purity and identify. This contract also provides storage for radiolabeled materials and distributes these materials as directed by National Cancer Institute staff.

NAPROTECH RESEARCH LAB (N01-CM9-7562) (Formerly N01-CM6-7705)

The objective of this contract is to search through the literature published worldwide on natural products and related fields and provide the Project Officer pertinent information required for the evaluation and acquisition of new and novel compounds that may have biological activity useful in the treatment of cancer. Reports of biological activity of extracts of plants, animals bacteria, fungi and marine organisms are also provided. This is an important contract for the acquisition of new agents for the DTP anticancer screening program. This is a one year contract, but the government may, by unilateral modification, require the contractor to perform for a second and/or third year.

NATIONAL ACADEMY OF SCIENCES (N01-CM5-7644)

This contract Task Order serves to develop standards for animal care and maintenance; shipping standards for the various species of laboratory animals, standards for nomenclature used to identify stocks and strains of laboratory animals; standards for animal maintenance in the research laboratory; and laboratory animal procurement standards. These standards are formulated by an Ad Hoc committee whose membership represent commercial animal production colonies, governmental and academic institutions, and non-profit research institutions.

NEW MEXICO STATE UNIVERSITY (N01-CM6-7974)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

NEW MEXICO STATE UNIVERSITY (N01-CM8-7278)

This contract provides for chemical re-synthesis of a variety of organic or inorganic compounds that have been identified as meriting investigation. These compounds are unobtainable from the original sources and are needed for biological evaluations. The types of compounds to be synthesized may include nitrogen-, oxygen-, and sulfur-containing heterocycles, peptides, metal complexes, nucleosides, anti-sense nucleic acids, etc.

NEW YORK BOTANICAL GARDEN (N01-CM6-7924)

This contractor is undertaking plant collections in Central and South America with emphasis on the tropical rain forest areas. The objective is to collect 1,500 plant samples per year for submission to NCI for extraction and screening against a panel of human cancer cell lines and for anti-HIV activity. Collections are currently ongoing in twelve Central and South American countries, and over 4,000 samples have been collected, including many medicinal plant samples from Belize.

NORTHWESTERN UNIVERSITY (N01-CM8-7257)

This contract is designed to monitor and maintain genetic control of tumor strains and inbred mouse stocks. This service was established to assure continuous control of the biological materials used in program studies. The contractor carries out the service by performing skin grafts and antigenic studies of mouse strains to assure their continuous genetic integrity and histocompatibility with other sublines maintained in counterpart genetic production centers.

OHIO STATE UNIVERSITY RESEARCH FOUNDATION (N01-CM6-7903)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of the Phase I trials of new agents. Task Orders are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was successfully completed and terminated on April 13, 1989.

OHIO STATE UNIVERSITY RESEARCH FOUNDATION (NO1-CM9-7619)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of the Phase I trials of new agents. Task Assignments are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance.

ORI, INC. (NO1-CM6-7909)

This is a "fast response" service contract, intended to provide computer programming support for the various and often urgent needs arising from the DTP drug testing program. Accordingly, in the past, the contractor participated in the design of DTP's new relational database of biological test results, designed and implemented computer generated graphical cancer and AIDS supplier report forms, maintained the HP laser printer, created a PC-based system to facilitate interactive viewing of AIDS and cancer data, etc. The contract is operated through a task-order mechanism.

PATHOLOGY ASSOCIATES, INC. (NO1-CM8-7258)

This contract provides pathology and veterinary services to the Toxicology Branch to support the preclinical toxicological evaluation of drugs for cancer and AIDS. In addition to a pathology materials repository, this contract is utilized to perform pathology quality assurance review of completed studies and studies in progress; pathology support in the form of site visits, photomicrography, slide preparation, performing necropsies, and histopathologic diagnosis of lesions; veterinary support such as site visits, the development of special surgical procedures, and instruction in these procedures; and equipment storage, maintenance and shipment. This is the second year of a five year contract.

PHARM-ECO LABORATORIES, INC. (NO1-CM6-7928)

This service preparative contract provides for the resynthesis of a variety of compounds required for clinical evaluation in Phase II and III trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality

required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

PHARM-ECO LABORATORIES, INC. (NO1-CM6-7933)

This service preparative contract provides for the resynthesis of a variety of compounds required for clinical evaluation in Phase II & III trials as potential anti-AIDS agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

POLYSCIENCES, INC. (NO1-CM6-7977)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

POLYSCIENCES, INC. (NO1-CM8-7271)

This Master Agreement for Master Agreement Orders in the large-scale isolation of anti-AIDS agents from natural sources was established to permit the isolation and purification of active materials from organisms, such as plants and marine invertebrates, for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. As yet, no project has been assigned to the contractor. Responsibility for supervision of this contract was recently transferred from the Natural Products Branch to the Pharmaceutical Resources Branch.

POLYSCIENCES, INC. (NO1-CM9-7621) (Formerly NO1-CM6-7707)

This Master Agreement for Master Agreement Orders in the large-scale isolation of antitumor agents from natural sources was established to permit the isolation and purification of active materials from organisms, such as plants and marine invertebrates, for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. The contractor is currently working on a task to isolate taxol, NSC 125973, from the bark of Taxus brevifolia. Responsibility for supervision of this contract was recently transferred from the Natural Products Branch to the Pharmaceutical Resources Branch.

PROGRAM RESOURCES, INC. (NO1-C07-4102)

This contractor is located at the Frederick Cancer Research Facility in Frederick, Maryland, and is divided into the following projects:

AIDS Chemical Prep Laboratory (Project 76611). This project is responsible for the appropriate weighing, solubilization, partial chemical characterization and the preparation of multiple aliquots of synthetic and natural product materials prior to their testing for ability to inhibit the growth of HIV in vitro, as well as in vivo when the assay procedures (for the latter) are established.

AIDS Computer Support (Project 76683). This project provides computer read-out and data processing support for synthetic and natural products acquisitions, extractions, Prep Lab, in vitro and in vivo testing, i.e. all drug evaluation aspects of the Developmental Therapeutics Program.

AIDS In Vitro Drug Screen Lab (Projecct 76612). This project provides research and development capacity, the facilities and the staff to develop the necessary technology, and implement the screening of large numbers of anti-HIV compounds annually in a safe, semi-automated system. Additionally, it serves as a conduit for the transfer of screening technologies to other laboratories involved in anti-HIV screening.

AIDS In Vivo Drug Testing Lab (Project 76613). This project supports a research and development effort to establish systems whereby various drugs and natural products could be tested in vivo for their anti-HIV activity. This is envisioned as a follow-up assay for compounds shown as active in vitro.

AIDS Natural products laboratory (Project 76682). This project provides crude natural product extracts (plant and marine) in support of the in vitro and in vivo testing programs.

AIDS Natural Products Repository (Project 76681). This project provides freezer storage space and cataloging support for natural product collections and for extracted natural products.

AIDS Protease Inhibition (Project 76667). This project provides support for the development and implementation of a mechanistically-oriented screen to measure capabilities of compounds to inhibit viral protease production.

AIDS Renovation-Building 431 (Project 90425). This project provides for the renovation of laboratory space for expanding natural product extractions and the in vitro AIDS screen and for an interim natural product isolation and identification laboratory.

Biological Laboratory Training Program (Project 61305). Support is provided for students from Frederick Community College (FCC) who work a minimum of 20 hours per week for DTP projects. Support includes tuition, books, guest lecturer fees, and some laboratory expenses when the project is not clearly defined.

Biological Testing Branch (BTB) Supplies (Project 58201). This project permits BTB personnel to utilize the PRI warehouse for obtaining office supplies.

Biological Testing Branch (BTB) Support (Project 76641). Provides for two Information Specialists in the Office of the BTB.

Computer Building (Project 90428). This project provides for the erection of a modular building to house the Developmental Therapeutics Program dedicated computer and for supportive contractor personnel.

Computer Support (Project 76629). This project provides computer read-out and data processing support for synthetic and natural products acquisitions, extractions, Prep lab, in vitro and in vivo testing, i.e. all drug evaluation aspects of the Developmental Therapeutics Program.

DTP Administrative Modular Building (Project 90426). This project provides for the erection of modular office space for Developmental Therapeutics Program personnel assigned to the FCRF.

DTP Chemical Prep Lab (Project 76625). This project is responsible for the appropriate weighing, solubilization, partial chemical characterization and the preparation of multiple aliquots of natural
product and synthetic materials, prior to evaluating their ability to inhibit growth of a wide variety of human tumor cell lines utilizing in vitro and in vivo testing systems.

DTP Publications, Etc. (Project 58211). This project permits Developmental Therapeutics Program personnel to use the publications, graphics, and library services at the FCRF.

Fermentation Pilot Plant Renovation (Project 90427). This project provides for the renovation of Building 472 which will be utilized for expanded production of selected natural product materials.

Genetic Monitoring (Project 76661). Monitors all Biological Testing Branch contract rat colonies for genetic purity. In addition, monitors the starts received from VRB-NIH, both pre and post rederivation.

Gut Flora Monitoring (Project 76662). Receives thirty animals weekly from one of our Primary Genetic Centers. The animals originate from our isolator colonies at the Genetic Centers, and are monitored for gut flora and possible virus contamination. The animals are sent weekly on a schedule made up the by Biological Testing Branch.

Human Tumor Procurement and Preparation (Project 76642). Tumors are obtained as cell lines in various stages of development and are also obtained as xenografts from surgical specimens. Tumors are then adapted for the in vitro/in vivo DTP screening effort.

In Vitro Cell Line Development (Project 76624). The project provides in vitro cell line development support. The Developmental Therapeutics Program goal is to develop 60-100 well-characterized cell lines with available patient history as soon as possible.

In Vitro Cell Line Screen - Natural Priducts (Project 76623). project Provides in vitro screening support, primarily for natural products. Capacity is expected to reach an annualized level of approximately 10,000 tests in FY '89. Approximately 60 human tumor cell lines are included in each test.

In Vitro Cell Line Screen - Synthetic Compounds (Pprject 76622). Provides in vitro screening support, primarily for synthetic compounds. Capacity is expected to reach an annualized level of approximately 10,000 tests in FY '89. Approximately 60 human tumor cell lines are included in each test.

In Vitro Cell Line Support (Project 76621). Purchases supplies and equipment which are used jointly by the two in vitro cancer cell line screening projects. Joint purchases are made to maximize the uniformity of testing procedures.

In Vivo Model Development and Testing (Project 76602). Human tumor cell lines are developed for use as models for in vivo drug evaluations and for selected testing of in vitro "actives".

Natural Products Extractions (Project 76652). Provides crude natural product extracts (plant and marine) in support of the in vitro and in vivo testing programs.

Natural Products Repository (Project 76651). Provides freezer storage space and cataloging support for natural product collections and for extracted natural products.

Operational Support for Harlan/Sprague Dawley (Project 27181). Provides Work Orders and Shared Service type functions for the Harlan Sprague Dawley Animal Production contract at Frederick. It serves as a method for paying PRI for services performed at the Animal Production Area at Frederick.

Program Development Research Group (PDRG) Laboratory Support (Project 58300. Provides service support, including materials, supplies, and equipment purchases for the PDRG intramural laboratory.

Production of Bryostatin (Project 76654). This is a shared service project which provides support for the production of Bryostatin.

Renovations for Animal Production Area/Harlan Sprague Dawley (Project 90450). Permits PRI to perform renovations for the Animal Production Area utilizing subcontractors.

Renovations for Building 431 (Project 90420). t 90420). Provides for the renovation of laboratory space for expanding natural product extractions and the in vitro AIDS screen, and for an interim natural product isolation and identificaiton laboratory.

Renovations of Five Animal Production Area Buildings (Project 90429). Provides for the renovation of laboratory space for in vivo model development and testing of selected agents from the in vitro cancer and AIDS screens.

Rodent Serology Monitoring (Project 76665). Receives serum samples from animal contractors and research laboratories to test for viral contaminants monthly. The scheduling of the serum samples is done by the Biological Testing Branch Project Officer.

Screening Support for Natural Products Isolation (Project 76666). Provides limited screening support for natural products isolation.

Taxol Isolation (Project 76653). This is a shared service project which provides taxol isolation capabilities from crude raw material.

Tumor Bank (Project 76601). This segment has as its major goal the maintenance of approximately 20,000 frozen tumor vials. Furnishes needed tumors to the various Developmental Therapeutics Program laboratories, as well as to other research institutions, both domestic and foreign. Tumors are supplied both in vivo and in vitro. This contract is located at the Frederick Cancer Research Facility.

PURDUE RESEARCH FOUNDATION (N01-CM6-7699)

The objectives of this contract are to design and synthesize the following: (1) congeners of anticancer lead compounds to enhance the activity or broaden the antitumor spectrum; and (2) prodrugs that are chemically altered transport forms of the lead compound. The chemical modifications will aim at improving biological and pharmaceutical properties including (a) water-solubility; (b) hydrolytic stability; and (c) spectrum of activity and specificity. In addition, the contract provides for the modification of compounds of natural origin and synthesis of heterocycles with improved antitumor activity and reduced toxicity. These modifications may range from partial structures to structural analogs.

PURDUE RESEARCH FOUNDATION (N01-CM8-7268)

This contract is for chemical synthesis and drug design of a variety of compounds for evaluation as potential anti-AIDS agents. The specific objectives of this project are: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that possess elements of both congener and prodrug; (c) to synthesize compounds related to products of natural origin and other related heterocycles; and (d) to synthesize anti-sense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

RAYLO CHEMICALS, LTD. (N01-CM6-7975)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

RESEARCH TRIANGLE INSTITUTE (N01-CM6-7970)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

RESEARCH TRIANGLE INSTITUTE (N01-CM7-3714)

This is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the Developmental Therapeutics Program as potential antitumor agents. The contractor determines identity and purity of the compounds by appropriate methods. The contractor also determines solubility, stability and other physical-chemical properties to aid in formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality asurance monitoring.

RESEARCH TRIANGLE INSTITUTE (N01-CM8-7227)

This contract provides for the synthesis of radiolabelled anti-AIDS compounds for use in preclinical pharmacological and clinical studies. The materials are analyzed for purity and identity by autoradiography assay, etc. This contract also provides storage facilities for labeled materials and distributes labeled compounds as directed.

RESEARCH TRIANGLE INSTITUTE (N01-CM9-7561)

This contract provides for the synthesis of radiolabelled anticancer chemicals and drugs for use in preclinical pharmacological and clinical studies. The materials prepared are not available from commercial sources. All materials are analyzed for purity and identity by autoradiography assay, etc. This contract also provides storage facilities for labeled materials and distributes labeled compounds as directed by National Cancer Institute staff.

RICERCA, INC. (N01-CM9-7613)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors.

SIMONSEN LABORATORIES (N01-CM9-7624) (Formerly N01-CM6-7917)

This contract is a Primary Genetic Center. Rederived associated flora pedigreed starts are supplied to this contract for propagation into isolator maintained foundation colonies. These foundation colonies supply breeders on a weekly basis to pedigreed expansion colonies which support the production colonies. Both the pedigreed expansion and production colonies are maintained in a barrier room environment. Offspring from the production colonies are used for hybrid production and the many NCI research activities.

SOUTH FLORIDA, UNIVERSITY OF (N01-CM6-7973)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

SOUTHERN CALIFORNIA, UNIVERSITY OF (N01-CM9-7620)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of the Phase I trials of new agents. Task Assignments are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on *in vitro* screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance.

SOUTHERN RESEARCH INSTITUTE (N01-CM4-7646)

This contract is for *in vivo* testing at a level of 24,000 L1210 equivalents. The majority of testing is conducted utilizing tumor systems and protocols of the secondary tumor panel. Minimum testing is conducted in the P388 prescreen, which is being phased out. In depth *in vivo* studies for the characterization of potential new *in vivo* tumor models are conducted at the request of the

Project Officer. This contract utilizes both conventional and nude mice. This contract was scheduled for termination September 30, 1988; however, it was extended until Fenruary 1989 and has now terminated.

SOUTHERN RESEARCH INSTITUTE (NO1-CM6-7905)

Preclinical pharmacology studies of antitumor/anti-AIDS agents under development by DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data as well as the efficiency of the Phase I trials of new agents. Task Orders are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was successfully completed and terminated on April 30, 1989.

SOUTHERN RESEARCH INSTITUTE (NO1-CM6-7911)

This contract was awarded in order to assist the Biological Testing Branch in the monitoring and quality control of existing human and murine tumor lines, as well as the host animals. The development of new human tumor lines, parallel to the disease-oriented in vitro prescreen, is conducted under this contract. Suggestions regarding the refinement of existing protocols are made as deemed necessary, and the cell kinetics for current tumor models are elucidated. This contract terminated February 1989.

SOUTHERN RESEARCH INSTITUTE (NO1-CM6-7968)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

SOUTHERN RESEARCH INSTITUTE (NO1-CM7-3726)

The major objective of this contract is to optimize the antitumor activity of agents identified by primary screens. To meet this objective, studies using various in vivo experimental tumor models are conducted in which drug concentration, and exposure time of the tumor cells and host to the drug are varied. Results are interrelated with pharmacokinetic, toxicologic and biochemical information to devise and recommend treatment strategies for clinical trial. Other objectives are to explore new therapeutic approaches, and to more fully assess the therapeutic potential of a new drug by conducting experiments against advanced-stage tumors and tumors at different sites, and by determining drug-resistance profiles. This contract is being recompeted.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7229)

This contract is part of the new Developmental Therapeutics Program initiative for AIDS drug development. A wide variety of organic/ inorganic compounds that have been identified by the DTP as meriting investigations are synthesized in 0.1 to 5 gram quantities. The compounds prepared are unobtainable from the original source and are needed for biological evaluations. All synthesized compounds are characterized as to identity and purity.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7235)

This contract is for the performance of special preclinical toxicology studies of potential therapeutic agents for treating AIDS and serves to bridge the gap between initial screening and full-scale, protocol toxicology studies. The types of studies performed under the contract can include: range-finding studies to determine the drug's maximum tolerated dose; plasma and CSF drug level determinations; myelosuppressive potential of cultured bone marrow stem cells; and intracellular drug pharmacology and biochemical studies. This is the first year of a three year contract.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7237)

This contract "Primary Screening of Compounds Against HTLV-III/LAV (Human AIDS Virus)" provides a capacity of 10,000 tests per annum. This contract is used for the testing of pure synthetic compounds (as opposed to crude natural products), and is the primary vehicle to obtain this testing.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7259)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 120 hours; and pharmacokinetics. The data from these studies are used to prepare attachment 6a for the INDA. This is the second year of a five year contract.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7274)

The objectives of this project are to optimize the in vivo antiviral activity, and evaluate the therapeutic potential, of compounds that are known to inhibit the growth and/or cytopathic effects of the human immunodeficiency virus (HIV). These objectives are achieved by conducting a series of specialized in vitro and in vivo experiments using murine retroviral models which possess one or more properties of the syndrome produced by HIV infection. Tasks include evaluation of the influence of drug schedule and route of administration on antiviral activity and determination of anti-viral activity of formulated products. Results from this project will be interrelated with pharmacologic and toxicologic information to devise and recommend treatment strategies for clinical trial.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7285)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the contractor through a Task Assignment mechanism. These studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) in vitro stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following i.v. bolus and infusion dosing in mice, rats, and dogs; (4) identification of drug metabolites. Data obtained in these investigations is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of drug in biological fluids. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Pre-clinical pharmacology studies of potential anti-AIDS agents are generally performed in parallel with, and are designed to aid in the interpretation of preclinical toxicology evaluations. Together, these investigations provide required data for INDA filing as well as a rational basis for the clinical Phase I starting dose and dose escalation scheme.

SOUTHERN RESEARCH INSTITUTE (NO1-CM9-7553)

This contract entitled "Quality Control and Model Development in Rodents and Tumor Cells" has as its primary objective the development and quality control of in vivo human tumor models to complement the in vitro disease-oriented human tumor cell line screen. In vivo models are being developed which are suitable for the screening of lead compounds generated by the in vitro program, and these models are monitored for both the integrity of the tumors and of the athymic mice used for this testing. This contract also provides support for in vitro cell line development and in vivo testing of "actives" from the in vitro screen.

SOUTHERN RESEARCH INSTITUTE (NO1-CM9-7574)

This contract is for the performance of preclinical toxicology studies of potential therapeutic agents for treating AIDS. These preclinical studies primarily are designed to relate target organ toxicity to plasma drug concentration and in vitro efficacy. Protocols include pharmacokinetic and bioavailability studies in rodents and dogs, cerebrospinal fluid penetration by

the drug in dogs, continuous intravenous infusion toxicity studies in dogs and 28 day multiple dose toxicity studies in dogs and rodents. The data from these studies are used to prepare attachment 6a for the INDA. This is the first year of a three year contract.

SPRINGBORN LIFE SCIENCES, INC. (NO1-CM8-7256)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 120 hours; and pharmacokinetics. The data from these studies are used to prepare attachment 6a for the INDA. This is the second year of a five year contract.

SRI INTERNATIONAL (N01-CM6-7864

This is one of two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phass of the Developmental Therapeutics Program as potential anti-AIDS agents. The contractor determines identity and purity of the compounds by appropriate methods. The contractor also determines solubility, stability and other physical-chemical properties to aid in formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring.

SRI INTERNATION (N01-CM6-7969)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

SRI INTERNATIONAL (N01-CM7-3715)

This is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the Developmental Therapeutics Program as potential antitumor agents. The contractor determines identity and purity of the compounds by appropriate methods. The contractor also determines solubility, stability and other physical-chemical properties to aid in formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and

nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring.

STARKS ASSOCIATES, INC. (N01-CM6-7798)

This service preparative contract provides for the resynthesis of a variety of compounds required for toxicology and clinical evaluation in Phase I trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

STARKS ASSOCIATES, INC. (N01-CM6-7926)

This service preparative contract provides for the resynthesis of a variety of compounds required for the clinical evaluation in Phase II and III trials as potential antitumor agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

STARKS ASSOCIATES, INC. (N01-CM6-7934)

This service preparative contract provides for the resynthesis of a variety of compounds required for the clinical evaluation in Phase II and III trials as potential anti-AIDS agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

STARKS ASSOCIATES, INC. (N01-CM6-7978)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors. This contract will expire during this fiscal year.

STARKS ASSOCIATES (N01-CM8-7231)

This contract to a small business is for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for anti-AIDS development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the original

investigators, are also synthesized by this mechanism. This mechanism may also be used for the resynthesis of a limited number of compounds of interest to intramural scientists.

STARKS ASSOCIATES, INC. (N01-CM9-7569)

This contract is in support of the Drug Synthesis and Chemistry Branch's fundamental responsibility to acquire selected novel synthetic and pure natural product compounds for evaluation as potential anticancer and anti-AIDS agents - the initial step in the National Cancer Institute's drug development process. The major focus of this contract is the active solicitation, acquisition, documentation and management of the flow of approximately 10,000 compounds per year of diverse structural and biological types. These compounds are selected by the Drug Synthesis and Chemistry Branch from a much larger pool of compounds provided through this contract in quantities adequate for the primary screens. This contract also acquires a significant proportion of the larger samples needed for secondary screening of the many new leads that are identified. In addition, this project continually monitors the scientific literature in order to provide the Branch with (1) a list of compounds for potential acquisition and testing in the NCI's screening programs and (2) listings of key publications in the areas of cancer and AIDS chemotherapy.

STATE UNIVERSITY OF NEW YORK RESEARCH FOUNDATION (N01-CM6-7698)

The objectives of this contract are to design and synthesize the following: (1) congeners of anticancer lead compounds to enhance the activity or broaden the antitumor spectrum; and (2) prodrugs that are chemically altered transport forms of the lead compound. The chemical modifications will aim at improving biological and pharmaceutical properties including (a) water-solubility; (b) hydrolytic stability; and (c) spectrum of activity and specificity. In addition, the contract provides for the modification of compounds of natural origin and synthesis of heterocycles with improved antitumor activity and reduced toxicity. These modifications may range from paritial structures to structural analogs.

STATE UNIVERSITY OF NEW YORK RESEARCH FOUNDATION (N01-CM8-7216)

This contract is for chemical synthesis and drug design of variety of compounds for evaluation as potential anti-AIDS agents. The specific objectives of this project are: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that possess elements of both congener and prodrug; (c) to synthesize compounds related to products of natural origin and other related heterocycles; and (d) to synthesize anti-sense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

SYNTHETIC, INC. (N01-CM9-7616)

This is one of the 15 Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds which have been identified by program for development. Unique compounds from the literature, with reported biological activity which cannot be obtained in sufficient quantities from the

original investigators, are also synthesized by this mechanism. This mechanism is also used for the resynthesis of a limited number of compounds of interest to intramural scientists, and potential radiosensitizers and radioprotectors.

TACONIC FARMS (NO1-CM5-7730)

This Rodent Production Center contract produces athymic nude mice under maximum barrier conditions.

TECHNICAL RESOURCES, INC. (NO1-CM8-7249) (Formerly NO1-CM6-7907)

This Developmental Therapeutics Program contract provides a Program-wide resource for support services to the extramural preclinical anticancer and anti-AIDS drug discovery and development efforts. The services include (1) support to the functions of decision-point committees; (2) planning and logistical management for DTP-sponsored conferences, seminars and workshops, including preparation of proceedings; (3) maintenance of files for the grants, contracts and NCDDG programs; (4) special reports and other Program-related documents; (5) graphics, slides and prints on a rapid turnaround basis; and (6) a variety of miscellaneous tasks related to the planning and operational phases of the total DTP effort.

TEXAS A&M RESEARCH FOUNDATION (NO1-CM3-7536)

This contract monitors the genetic purity of the strains produced at the Genetic Centers and Rodent Production Centers. The testing is done by checking biochemical markers, and animals are sent for monitoring on a weekly basis scheduled by the Project Officer.

UTAH, UNIVERSITY OF (NO1-CM6-7863)

This contract carries out dosage form development studies leading to an acceptable injectable dosage form on compounds with activity versus HIV. These studies involve solubility assessments, determination of pH versus stability profiles, preparation of pilot scale batches, and evaluation of the stability of the product under simulated use conditions. The contractor has experience with several methods of improving drug solubility, including complexation and preparation of prodrugs that is being applied to resolve difficult formulation problems. This contract is being recompeted.

VANGARD LABS (NO1-CM8-0065)

The Pharmaceutical Resources Branch provides pharmaceutical support to the National Institute of Allergy and Infectious Diseases for their ATEA 019 clinical trial. This resource contract provides for the double blind blister packaging of the active and placebo capsules used in this study. This is presently the largest AIDS protocol under study.

VERMONT REGIONAL CANCER CENTER (NO1-CM8-7286)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the contractor through a Task Assignment mechanism. These

studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) in vitro stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following i.v. bolus and infusion dosing in mice, rats, and dogs; (4) identification of drug metabolites. Data obtained in these investigations is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of drug in biological fluids. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Pre-clinical pharmacology studies of potential anti-AIDS agents are generally performed in parallel with, and are designed to aid in the interpretation of preclinical toxicology evaluations. Together, these investigations provide required data for INDA filing as well as a rational basis for the clinical Phase I starting dose and dose escalation scheme.

VSE CORPORATION (N01-CM5-7654)

This service contract provides for operation and maintenance of the Developmental Therapeutics Program Biological Data Processing System. The system provides support for acquisition of biological testing data produced by DTP's anti-cancer and anti-viral drug screens. Both of these activities currently rely on automated in vitro test systems which are being developed to allow screening of many thousand samples per year. This contract provides software support at the laboratory level for data acquisition, as well as support at the mainframe level for maintenance of data files. Support is also provided for acquisition and maintenance of in vivo drug testing data.

WORLD BOTANICAL ASSOCIATES (N01-CM8-7281)

This Master Agreement for Master Agreement Orders for the collection of terrestrial plants for anti-AIDS drugs was established to allow the collection of sufficient material to permit the isolation of active compounds from the plants for use in preclinical development and clinical trials. This Master Agreement Order mechanism enables the prompt solicitation and award of projects to be conducted in this area from a pool of qualified contractors. As yet, no project has been assigned to the contractor.

Z, INC. (N01-CM7-3720)

The objective of this small business contract is to perform a variety of computer searches such as full structure searches, substructure searches and data item searches in support of the DTP program. The contractor utilizes several data bases such as DIS, DARC, Questal, NLM, and Dialog. Another task under this contract is the development of chemical names for compounds of interest. In addition, the contractor also performs searches for crude natural products on a selected basis.

RADIATION RESEARCH PROGRAM

ALLEGHENY-SINGER RESEARCH CORPORATION (NO1-CM8-7245)

This contract is expected to develope new or modify existing criteria, guidelines and procedures for the proper use of the equipment representing the major heat generating modalities (radiofrequency, microwave and ultrasound) and the ancillary equipment necessary for the treatment of cancer with heat as the technology advances. The criteria and guidelines developed and/or modified will be utilized to conduct a quality assurance and assessment program in hyperthermia. This contractor is expected to continue to implement and conduct such a program during the lifetime of the contract. This contract was recently recompeted.

AMERICAN COLLEGE OF RADIOLOGY (NO1-CM8-7275)

The American College of Radiology (ACR), Philadelphia, was awarded a four-year contract to assess the state-of-the-art of radiation therapy and the quality of patient care in the U.S. Purpose of the contract is to extend our knowledge about patient outcome as a function of the treatment process in a number of tumor sites. The survey of approximately 1200 treatment centers will focus on sites examined in previous PCS and extend the analysis to some new tumor sites. Data collected under this contract will provide a total census of megavoltage facilities in the U.S. and Puerto Rico and document trends since 1973. Analysis of patient outcome will be used in educational programs designed to improve the quality of patient care and to document best current management for specific tumors and disease sites.

ANALYSIS AND SIMULATION, INC. (N43-CM9-7603) (SMALL BUSINESS INNOVATION RESEARCH Program

Analysis and Simulation, Inc. Buffalo, NY, has been funded with a Phase I SBIR contract to develop a prototype software system for the management of data related to clinical investigational protocols in radiation therapy. The system will be designed to facilitate information access and retrieval, organize data handling and provide expert advice, including provisions for exploiting existing data base system, such as the NCI's Physician Data Query (PDQ) data base.

CALIFORNIA, UNIVERSITY OF (NO1-CM9-7315)

The University of California, Los Angeles, (UCLA) is a member of the Neutron Therapy Collaborative Working Group (NTCWG), a consortium of three institutions funded by NCI to carry out neutron therapy clinical trials. UCLA participates in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy. Four clinical studies are currently under investigation by the NTCWG in several anatomical sites: head and neck, prostate, lung and sites of radioresistant histologies, such as melanoma, renal cell carcinoma and sarcomas of the soft tissue and bone.

CHEUNG LABORATORIES, INC. (N44 CM7-7838) (SMALL BUSINESS INNOVATION RESEARCH Program)

In a Phase I study, this contractor demonstrated the feasibility of using phase and amplitude controlled multiple microwave applicators for improving the power disposition patterns, i.e., the specific absorption rate (SAR), when inducing hyperthermia in deep seated tumors by electromagnetic means. In the Phase II study, the contractor will perform dosimetry studies using his microstrip applicators and tissue equivalent phantoms. Heating patterns will be measured in the phantoms at various frequencies. The power generating system will be automated to control the phase and amplitude of the applied power to each applicator to achieve optimal heating patterns in desired locations. Software used will be developed and integrated into the system and the system tested in the clinical setting. This contract was terminated before completion for the convenience of the Government.

DOTY SCIENTIFIC, INC. (N44-CM9-7804) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract supports the development of electronic components for magnetic resonance imaging (MRI) systems in the form of low-noise pulse amplifiers with substantially lower initial costs and operating costs. In addition, a versatile, high power, high performance radiofrequency (rf) amplifier system has been developed and produced, and significant development progress has been made in protection systems, rf switches, pulse shaping circuits, and special design iron powder transformers for 20 kHz high power applications.

LASERGUIDE, INC. (N43-CM8-7261) (SMALL BUSINESS INNOVATION RESEARCH Program)

A NdYag (Neodymium-Yitrium-Luminum-Garnet) laser based system for interstitial and external beam hyperthermia for the treatment of cancer will be developed. Such a system could provide a very cost effective method which does not have the problems of the present microwave and radiofrequency systems. It would provide local hyperthermic heating which would not have constructive/destructive interference problems and which can be monitored and controlled by the use of an inexpensive microthermal couple system.

Phase I studies will include (1) determination of the optimum NdYag wavelength to be used (1.06 or 1.32 microns), (2) what is the actual delivered power required to raise a given volume of tissue to $<45^{o}$? (3) what is thermal distribution generated in vivo (rat model)? and (4) evaluation of thermal couple monitoring and feedback control mechanism for the system. The information obtained in Phase I will be used to develop a more complete system in Phase II aimed at more advanced animal and clinical testing. This Phase I contract was sucessfully completed and a Phase II application has been submitted.

LUXTRON CORPORATION (N44-CM7-7841) (SMALL BUSINESS INNOVATION RESEARCH Program)

The overall objective of this project is to develop a system for fast, accurate, on-line measurement and control of tissue temperature during microwave-induced interstitial hyperthermia as used in cancer therapy. Phase I has demonstrated the feasibility of instrumenting an interstitial catheter with multiple rf-immune fiberoptic sensors located in the wall of the catheter without significantly increasing catheter size. This design leaves the bore free for insertion of the microwave antenna, places the sensors in better thermal contact with the tissue being heated, and makes possible an on-line assessment of the temperature distribution along the length of the antenna during treatment. Phase II has two major subgoals, namely; (1) further improvement of the catheter and sensor materials and design to allow a cost-effective volume fabrication and assembly; and (2) evaluation and preliminary development of a cost-effective instrument capable of supporting a large number of multisensor catheters. Prototype catheter evaluation will also include extensive animal and clinical testing. This contract will be successfully completed this year.

MEDICAL ADVANCES, INC. (N43-CM8-7260) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase I SBIR contract provides support for the development of novel techniques and designs of local coil networks to be used with magnetic resonance imaging (MRI) systems as an adjunctive technique for the diagnostic evaluation of breast cancer. Sequential parallel image acquisition (SPIA) techniques permit high resolution images of the breasts and axillary lymph nodes to be made in reduced total scan time.

MEGAVOLTAGE IMAGING (N43-CM9-7602) (SMALL BUSINESS INNOVATION RESEARCH Program)

Megavoltage Imaging, Gainesville, FL, is funded for a Phase I SBIR contract to design a prototype high-energy imaging device that can replace current methods of film portal imaging in radiation therapy treatment verification. The aim of the Phase I research is to demonstrate the feasibility of collecting projection data with a suitable ionizing liquid and producing an image within a few seconds.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NO1-CM5-7776)

This contractor was funded by the NCI for three and a half years as a member of the Interstitial Collaborative Working Group (ICWG), a consortium of three institutions, to develop recommendations and guidelines for brachytherapy. The group's recommendations include guidelines for calibration and dosimetry of clinical radioisotope sources, software packages for calculation, a quality assurance program, and the clinical use of interstitial radiotherapy. Several chapters describe tumor sites that would benefit from brachytherapy implant techniques. The recommendations document will be published in book form by Raven Press, Inc. and ready for distribution in the fall of 1989. This contract completed March 1989.

MICHIGAN, UNIVERSITY OF (NO1-CM6-7913)

The University of Michigan is a member of a consortium of three institutions, the Electron Collaborative Working Group (ECWG), funded to evaluate electron beam treatment planning, specifically to test new algorithms for calculating electron dose and to evaluate the role of tissue inhomogeneity corrections, error analysis and new treatment techniques. Recommendations and guidelines for the practice of electron beam radiotherapy will result from the ECWG efforts.

MICROWAVE MEDICAL SYSTEMS, INC. (N44-CM7-7821) (SMALL BUSINESS INNOVATION Program)

The goal of this SBIR contract is to develop a device for early detection of extravasation of intravenously administered cytoxic drugs using non-invasive microwave radiometry to monitor the fluid temperature at the injection site. Phase II of this contract began 15 September 1987 and is scheduled for two years. Progress in the first eighteen months of the contract is on schedule and satisfactory.

NORTH CAROLINA, UNIVERSITY OF (NO1-CM9-7565)

This contractor is part of a collaborative effort, the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded to develop new computer-based support systems that will provide new software tools to make three-dimensional treatment planning a routine activity. Specific tasks to be addressed include the automatic extraction of anatomical features from multiple CT images for the construction of three-dimensional volumes; aids in the tumor localization process, such as registration of images from multi-modality studies; display and evaluation of three-dimensional data for the selection of optimal treatment plans; software documentation developed by the RTPT-CWG will be published in the public domain for widest possible dissemination to the radiotherapy community.

NORTHERN CALIFORNIA CANCER PROGRAM (NO1-CM6-7868)

The capability for evaluating chemical compounds for radiation sensitizing and/or radiation protective properties is provided by this resource. Various physico-chemical parameters are determined on a large number of compounds representing a wide range of chemical structures. Compounds showing potential radiosensitizing or radioprotective characteristics will undergo *in vitro* testing to evaluate their cytotoxicity and degree of radiosensitization using mammalian cell cultures. Potential radiosensitizing compounds which appear to be superior to the standard - misonidazole - will be evaluated *in vivo*, using mice and/or rats. The effect of the compound in modifying the response to radiation will be measured in three different tumor systems (from the DCT panel of mouse tumor screens as stated in the Treatment Linear Array for Radiosensitizers), each measured by a separate endpoint. The endpoints will include: the regrowth delay of tumors, tumor cell survival and the modification of the radiation dose required for curing 50% of the tumors. All radioprotective compounds tested will be compared with the standard - WR-2721. The contract also provides for the option of assessing potential long-term tissue injury by performing histopathology on a limited number of tissues and

compounds. This contract should provide new radiosensitizers and radioprotectors or leads in developing new types (classes) of radiation modifying compounds.

RADIATION MONITORING DEVICES, INC. (NO1-CM6-7807) (SMALL BUSINESS INNOVATION RESEARCH Program)

The Radiation Monitoring Devices (RMD) completed the development and improvement of the photovoltaic CdTe radiation sensor. The hardware and software for the detector were developed and fully operational. An operational prototype system was constructed and is being tested with clinical phantoms in collaboration with their medical and industrial consultants. The contractor will continue to evaluate the detectors. In Phase III, at no additional cost to the National Cancer Institute, the contractor will install the system at a hospital to evaluate the detector on human patients with breast cancer or diagnostic mammography.

RADIATION MONITORING DEVICES, INC. (N43-CM9-7606) (SMALL BUSINESS INNOVATION RESEARCH Program)

This SBIR Phase I contractor in Waterton, MA, will assemble a prototype sourcebook of treatment aids appropriate for radiation therapy. Purpose of the book is to keep treatment centers informed about therapy technologies and identify points of contact for obtaining the treatment devices. The Phase I feasibility study will conduct a pilot survey in the Boston area to develop and refine a categorization of types of treatment aids, to conduct a literature search, to identify sources of treatment aids, and to develop a scope of work and proposal for a full study.

RADIATION MONITORING DEVICES, INC. (N44-CM7-7842) (SMALL BUSINESS INNOVATION RESEARCH Program)

Under a Phase II SBIR research contract, Radiation Monitoring Devices, Waterton, MA, is developing a working prototype of a computer-controlled multi-leaf collimator which can conform to an arbitrarily shaped radiation treatment field. The device will fit on brackets currently in use on existing machines. Computer control of moving the leaves is provided through an IBM-XT, according to the portal shape entered with a digitizing tablet. The new collimator system will be well suited to treatment plans which require a number of treatment portals each day and portal-shaping during a dynamic treatment.

RADIATION MONITORING DEVICES, INC. (N44-CM9-7601) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase I SBIR contract will demonstrate the feasibility of developing a real-time, portal imaging system which can produce a good quality image within the first few seconds of treatment using a solid-state gamma ray detector. The feasibility study will determine the response of the CdTe detectors and optimize detector performance in the high energy, intense environment of an accelerator treatment beam. A conceptual design of a final prototype will be delivered under the Phase I contract.

SRI INTERNATIONAL (NO1-CM7-3708)

The objective of this contract between NCI, SRI International and Stanford University is the design, synthesis and biological evaluation of novel radiosensitizers. The primary focus of the work is the identification of leads other than electron affinicnitroimidazoles. Other types of compounds that are being investigated have different modes of action. These include inhibitors of the repair of potential lethal damage, shoulder modifiers and glutathione depleters.

TECHNISCAN, INC. (N44-CM9-7625) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase II SBIR contract provides for the design and development of an ultrasound imaging system which can eventually be optimized for clinical imaging of abdominal organs and, possibly, of the breast. This implementation will be based on highly advanced physics studies of the ways ultrasound energy propagates through biological tissues, which have been carried out at the University of Utah under ongoing grant no. R01 CA29728, with which the SBIR contract work has been closely integrated. Extensive use of computer algorithms which permit corrections to be made to each pixel element in the image for the absorption, refraction, and speed of ultrasound energy will enable the clinical user to achieve more accurate anatomical imaging of organs, as well as "quantitative" imaging of tissue properties.

TEXAS, UNIVERSITY OF, SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE NO1-CM5-7775)

M.D. Anderson Hospital (MDAH), University of Texas System Cancer Center, is a member of the Neutron Therapy Collaborative Working Group (NTCWG), a consortium of three institutions funded by the NCI to carry out neutron therapy clinical trials. MDAH participates in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy. Four clinical studies are currently under investigation by the NTCWG in several anatomical sites: head and neck, prostate, lung and sites of radioresistant histologies, such as melanoma, renal cell carcinoma and sarcomas of the soft tissue and bone.

TEXAS, UNIVERSITY OF SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (NO1-CM6-79140

The University of Texas System Cancer Center, M.D. Anderson Hospital, is a member of a consortium of three institutions, the Electron Collaborative Working Group (ECWG), funded to evaluate electron beam treatment planning, specifically to test new algorithms for calculating electron dose and to evaluate the role of tissue inhomogeneity corrections, error analysis and new treatment techniques. Recommendations and guidelines for the practice of electron beam radiotherapy will result from the ECWG efforts.

THERMAL TECHNOLOGIES, INC. (N44-CM7-7855) (SMALL BUSINESS INNOVATION RESEARCH Program)

In a Phase I study, this contractor combined two separate thermal probe methods to produce a simultaneous, integrated methodology for the local transient quantification of tissue thermal conductivity, diffusivity, and levels of tissue perfusion by a single measurement, eliminating the requirement for a no-flow calibration procedure. In Phase II, the contractor plans to initiate a program of instrument development and *in vitro* verification. In collaboration with other participating institutions, this thermally-based measurement instrument will be introduced into the clinical environment for a critical and timely evaluation of its usefulness in hyperthermia therapy. This contract will be successfully completed this year.

WASHINGTON UNIVERSITY (N01-CM6-7915)

Washington University, St. Louis, MO, is a member of a consortium of three institutions, the Electron Collaborative Working Group (ECWG), funded to evaluate electron beam treatment planning, specifically to test new algorithms for calculating electron dose and to evaluate the role of tissue inhomogeneity corrections, error analysis and new treatment techniques. Recommendations and guidelines for the practice of electron beam radiotherapy will result from the ECWG efforts.

WASHINGTON, UNIVERSITY OF (N01-CM9-7282)

The University of Washington, Seattle, is a member of the Neutron Therapy Collaborative Working Group (NTCWG), a consortium of three institutions funded by the NCI to carry out neutron therapy clinical trials. The contractor participates in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy. Four clinical studies are currently under investigation by the NTCWG in several anatomical sites: head and neck, prostate, lung and sites of radioresistant histologies, such as melanoma, renal cell carcinoma and sarcomas of the soft tissue and bone.

WASHINGTON UNIVERSITY (N01-CM9-7564)

Washington University at St. Louis is part of a collaborative effort, the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded to develop new computer-based support systems that will provide new software tools to make three-dimensional treatment planning a routine activity. Specific tasks to be addressed include the automatic extraction of anatomical features from multiple CT images for the construction of three-dimensional volumes; aids in the tumor localization process, such as registration of images from multi-modality studies; display and evaluation of three-dimensional data for the selection of optimal treatment plans; and on-line verification of patient treatment position with CT reconstructions. Code and software documentation developed by the RTPT-CWG will be published in the public domain for widest possible dissemination to the radiotherapy community.

WASHINGTON, UNIVERSITY OF (NO1-CM9-7566)

This contractor is part of a collaborative effort, the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded to develop new computer-based support systems that will provide new software tools to make three-dimensional treatment planning a routine activity. Specific tasks to be addressed include the automatic extraction of anatomical features from multiple CT images for the construction of three-dimensional volumes; aids in the tumor localization process, such as registration of images from multi-modality studies; display and evaluation of three-dimensional data for the selection of optimal treatment plans; and on-line verification of patient treatment position with CT reconstructions. Code and software documentation developed by the RTPT-CWT will be published in the public domain for widest possible dissemination to the radiotherapy community.

SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE BIOLOGICAL RESPONSE MODIFIERS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

INTRODUCTION

The Biological Response Modifiers Program (BRMP) is unique within the National Cancer Institute (NCI) in that it is a comprehensive program with both extramural and intramural basic and clinical research components charged with the investigation, development, and clinical testing of biological therapeutic agents that may alter host defenses or cancer cells directly in a way that could lead to tumor regressions in man. This unique structure facilitates the rapid movement of basic science and preclinical observations into clinical testing.

The extramural activities of the BRMP are planned, supervised, and monitored by the Biological Resources Branch (BRB) under the direction of Dr. Stephen P. Creekmore. The branch contains an active grants program and contracts supporting preclinical and clinical research throughout the biomedical research community. The BRB sponsors Phase Ia, Ib, and II clinical trials of biological agents looking not only for antitumor effects but also for the induction of changes in host biological functions that could be associated with an antitumor response. The BRB maintains a close working relationship with the Developmental Therapeutics Program (DTP) of the Division of Cancer Treatment (DCT) through the BRMP/DTP Working Group (BDWG) to coordinate preclinical development of biological agents with our sister program in preclinical drug development; and with the Cancer Therapy Evaluation Program (CTEP) through the BRMP/CTEP Working Group (BCWG) to coordinate the Phase II and III clinical development of biological agents with our sister program in clinical drug development. The BRB supervises an intramural contract-supported preclinical evaluation laboratory that studies the activity and mechanisms of action of biological agents in preclinical animal models that may have an important impact on the design and monitoring of human clinical trials, including the study of dose- and schedule-related variables, the biological and immunological parameters associated with in vivo tumor regression, and the detection of surrogate changes which might be measurable in man that correlate with the induction of a significant antitumor effect. The BRB serves as liaison with a large number of biotechnology companies and studies mechanisms of interacting with commercial interests and individual Government-supported researchers to foster biological therapeutic drug development. In addition, the BRB stimulates therapy-directed research through the sponsorship of scientific meetings and workshops, the establishment of standards for assessing the function of biological agents, the distribution of such standards, and the announcement of request for applications and program announcements to foster the development of particularly significant research topics.

In addition to laboratory research conducted under the auspices of the Associate Director, the intramural program of the BRMP consists of three basic science laboratories and one clinical branch: the Laboratory of Molecular

Immunoregulation (LMI), the Laboratory of Experimental Immunology (LEI), the Laboratory of Biochemical Physiology (LBP), and the Clinical Research Branch (CRB).

The LMI consists of three sections: the Immunobiology Section (IS) headed by Dr. Luigi Varesio, the Lymphokines Section (LS) under Dr. Frank Ruscetti, and a section created this year called the Cytokines Molecular Mechanisms Section (CMMS) led by Dr. William Farrar. The Laboratory Chief of the LMI is Dr. Joost Oppenheim, who also maintains an active research program. The LMI (1) investigates at the cellular, biochemical, and molecular level, the intercellular and intracellular processes that regulate host defense mechanisms; (2) studies the lymphokine/cytokine modulation of cellular functions in tumor cells and cells that participate in host defense; (3) evaluates the effects of biological agents on tumor cells, immunoregulatory pathways, and host defense mechanisms; and (4) identifies new agents that may modify the biology of tumor cells or host defense mechanisms.

The LEI is composed of three sections: the Cellular and Molecular Immunology Section (CMIS) under Dr. Howard Young, who was recruited this year to replace Dr. Craig Reynolds, the Leukocyte Differentiation Section (LDS) with Dr. John Ortaldo acting as interim head, and the Experimental Therapeutics Section (ETS) under Dr. Robert Wiltrout. The LEI laboratory chief is Dr. John Ortaldo. The LEI (1) conducts studies on biological agents and their application to the treatment of cancer; (2) studies the therapeutic use of cell-mediated immune effector mechanisms, lymphokines, cytokines, monoclonal antibodies, growth factors and their antagonists, other components of the host response, and chemotherapy alone and in combination in experimental tumor models; (3) studies the cellular and molecular mechanisms by which effector cells mediate antitumor activity; (4) studies the control of macrophage and lymphocyte activation; and (5) prepares new monoclonal antibodies or modifies existing antibodies as therapeutic agents or as probes to analyze cell differentiation and function, especially lymphoid and hematopoietic cells.

The LBP laboratory chief is Dr. Hsiang-fu Kung. The LBP (1) plans and conducts research on the genetic and biochemical events related to the development and expression of malignant phenotypes in human and animal cells; (2) applies skills in molecular biology, recombinant DNA technology and biochemistry to develop a comprehensive program to identify and isolate relevant genes and their products which relate to cell transformation, the control of cell growth and differentiation, and the regulation of host defenses; and (3) coordinates collaborative efforts between the BRMP and other intramural and extramural program elements in order to identify, clone, and isolate genes encoding molecules of potential value in the treatment of malignancy or the alteration of biological responses.

The CRB is being managed on an interim basis by Dr. Dan L. Longo, Associate Director, BRMP. The CRB (1) performs Phase Ia, Phase Ib, and Phase II clinical trials of selected biological therapeutic agents; (2) conducts innovative pilot studies integrating biologicals with other forms of treatment; (3) makes clinical correlations with extensive laboratory data collected to monitor the effects of biological agents on the host; and (4) coordinates the clinical research effort of the NCI in the treatment of lymphomas.

EXTRAMURAL PROGRAM ACCOMPLISHMENTS

There are extensive interactions with other programs with similar and potentially overlapping interests to facilitate smooth coordination of the development of biological therapeutic agents and strategies. As noted above, working groups with CTEP and DTP meet regularly to synergize and integrate activities. There is also a close working relationship with the Immunology and Tumor Biology Programs of the Division of Cancer Biology and Diagnosis and with the AIDS program of the National Institute of Allergy and Infectious Diseases (NIAID).

Grants Program

In FY89, there were 97 active grants with approximately $26.7 million in support. Of these, 66 were R01 grants, 11 were P01 program projects, 1 were R35 Outstanding Investigator Awards, 4 were new investigator awards, 3 were R13 conference grants, 8 were merit awards, and 4 were Small Business Innovative Research (SBIR) grants. There is a significant effort to develop monoclonal antibodies as effective therapeutic agents. The research effort focusses on melanoma, leukemia/lymphoma, colon cancer, renal cell cancer, and breast cancer. The studies underway are evaluating unconjugated antibodies and conjugates with nuclides and toxins with or without lymphokines to enhance the therapeutic effects. A number of grantees are evaluating methods to enhance the delivery of therapeutic agents to tumors. Improvements in adoptive cellular therapies are being developed. Sophisticated studies on the mechanism of action of cytokines and lymphokines are being supported and work to develop differentiation factors as therapeutic agents is also being conducted. The grants program is also supporting work to develop therapeutic agents using molecular biology tools. For example, hormone/toxin hybrids developed under the BRMP grants program are now being tested as therapeutic agents in melanoma and T-cell tumors. Other workers are studying the feasibility of molecular manipulations of oncogene expression or function and tissue-specific promoters and repressors to exert selective effects on neoplastic cells.

Requests for Application (RFAs)

In FY89, the BRMP issued a very interesting RFA soliciting grant applications on chronobiology and the influences of biorhythms on the efficacy and toxicity of cancer treatments. Although the response to the RFA was gratifying, the special study section convened to evaluate the applications gave only 2 of the 14 applications fundable priority scores. We have reviewed our experience with the RFA mechanism and have reached a conclusion that, as currently operated, it has not served the program and our grantees in the fashion for which it was designed. Of the 3 RFAs issued in FY88, only 4 of 47 applications receive fundable priority scores. In specific cases, we have found that the grant applications appear to fare better in the usual study sections than in the special study sections. Thus, we intend to favor the Program Announcement mechanism in the future.

Program Announcements

Grant applications are continuing to be submitted for the 15 Program Announcements previously issued. In FY89, BRMP and DTP jointly issued a Program Announcement: Specific Cancer Cell Targeting Using Molecular Genetic Technology. Other Program Announcements are being developed.

Contract Program

Two new preclinical contracts were the subject of Requests for Proposals (RFPs) in FY89: production of oligodeoxynucleotides and production of chimeric monoclonal antibodies. For each RFP, two or more Master Agreement Holders will be evaluated as qualified to provide materials for preclinical and clinical testing. A contract was awarded for the chelation of monoclonal antibodies for clinical studies involving imaging and therapy.

Phase Ia/Ib and Phase II Clinical Trials

The BRB maintained 26 clinical Task Order contracts with 15 extramural institutions for testing biological agents in Phase Ia/Ib clinical trials. The BRB has now established and funded 3 institutions with Phase Ia/Ib contracts for clinical studies of monoclonal antibodies, immunoconjugates, and other targeting molecules (Memorial Sloan-Kettering, University of Alabama, M.D. Anderson Hospital) and 4 institution with Phase Ia/Ib contracts for clinical studies of cytokines and immunomodulators (Cleveland Clinic, Memorial Sloan-Kettering, University of Southern California, University of Wisconsin). About $3.5 M was spent on clinical studies in FY89. Ongoing studies are evaluating a variety of monoclonal antibodies with one or more cytokines, radioimmunotherapy with antibody-isotope conjugates, potentially synergistic combinations of cytokines, adoptive cellular therapies, and colony-stimulating factors (CSFs) with and without chemotherapy.

The BRB clinical trials effort is closely linked with that of CTEP. Agents receiving initial evaluation in BRMP-sponsored Phase Ia/Ib and Phase II clinical trials are considered for late Phase II and Phase III studies administered by CTEP through their extensive clinical trials network of cooperative study groups and contractors. BRMP staff serve on the CTEP protocol review committee and CTEP staff serve on the BRMP operating committee. In addition, BCWG meetings are held weekly to coordinate the development of biological therapies.

Pharmaceutical Company Relationships

The BRMP has established relationships with nearly all of the biotechnology and pharmaceutical companies involved in the development of biological therapeutic agents. The BRB is responsible for fostering interactions between the Government and the private sector in the development of biological agents for cancer therapy through collaborations and formal research and development agreements.

New Initiatives

Plans are underway to establish a larger number of contracts to provide clinical amounts and grades of novel biological materials and to support innovative clinical investigators in the clinical testing of complex or sophisticated biological manipulations for the treatment of cancer and AIDS. In addition, efforts are underway to provide a centralized monitoring laboratory to assist in gleaning information on the biological effects of treatments that are part of studies not necessarily designed to address biological endpoints. For example, certain CTEP contractors are studying agents that might act either directly on tumors or on the host. The establishment of a central testing laboratory may assist in getting more information from these studies.

Preclinical Evaluation Laboratory

The Preclinical Evaluation Laboratory is operated as a contract with Program Resources, Inc. at the Frederick Cancer Research Facility. The Preclinical Evaluation Laboratory is investigating two major questions: (1) the most effective way to optimize the dose-intensity of chemotherapy programs with the use of strategies to protect the bone marrow and other organs from drug-related and/or radiation-related toxicity; and (2) the development of complex therapeutic interventions employing combinations of biological and other types of agents rationally combined and monitored with surrogate measures of host and/or tumor response that will facilitate the rapid translation of preclinical treatment advances into the clinic.

In the past year, the BRMP has initiated 2 clinical trials in man based upon findings in the Preclinical Evaluation Laboratory. Studies from this laboratory suggested that the optimal timing for a second course of chemotherapy after the use of CSFs was 7-10 days. We are now conducting a study to intensify the doses of nitrogen mustard and procarbazine in the MOPP regimen for Hodgkin's disease by using granulocyte macrophage-colony stimulating factor (GM-CSF) on days 8-14 and administering the next cycle of chemotherapy after a 7 day waiting period. In the work of the Preclinical Evaluation Laboratory on the synergistic antitumor effects of flavone-8-acetic acid (FAA) plus interleukin 2 (IL 2), it became clear that the practice of administering the FAA with alkalinization to prevent this weak acid from precipitating in the renal tubules was associated with a significant decrease in antitumor efficacy. Thus, we have amended our clinical trial to eliminate the alkalinization.

The laboratory has been exploring the role of a hemoregulatory peptide called HP 5b in marrow protection and restoration before and after administration of chemotherapy and radiation therapy. The monomeric form of the pentapeptide appears to have potent growth inhibitory effects on the marrow while the dimeric form stimulates the proliferation of marrow stem cells. The dimer is active when given in nanogram amounts orally in preventing the fatal myelotoxicity associated with the administration of cyclophosphamide or whole body irradiation. Studies on its mechanism of action are underway, as are efforts to determine whether its effects are additive with other myelostimulatory factors.

The laboratory has begun to evaluate the adoptive cellular therapy of human tumor xenografts in nude mice. Human monocytes activated with interferon-γ, IL 2, M-CSF, GM-CSF, and lipopolysaccharide are being evaluated for their capacity to produce antitumor effects against human ovarian and colon carcinomas in vivo. Efforts are also ongoing to evaluate the capacity of transforming growth factor β (TGF-β) to be used as an antitumor agent. The bryostatins, complex macrocyclic compounds isolated from a one-celled sea animal called Bugula nerititus, are capable of transducing signals through protein kinase C. For a wide variety of neoplastic cells, such signals result in growth arrest. The Preclinical Evaluation Laboratory is examining the therapeutic effects in vivo. The bryostatins also appear capable of stimulating marrow colony-forming units. Thus, their effects on in vivo hematopoiesis are also being evaluated.

Biological Response Modifiers Program Operating Committee (BRMPOC)

The BRMPOC is the steering committee for the BRMP extramural program. It reviews and prioritizes agents as to evaluation in preclinical models and in man. The BRMPOC makes recommendations to the DCT Decision Network Committee (DNC) about the prioritization of compounds for clinical development and in turn the recommendations of the DNC are implemented by the BRMPOC and BRB program staff.

BRMP-Sponsored Meetings

This year the BRMP has sponsored or co-sponsored three highly successful scientific meetings: a 2-day workshop on the "Establishment of Standards for Recombinant Human Cytokines" held on November 3-4, 1988 in Frederick, Maryland; a 3-day conference entitled, "Oligonucleotides as Antisense Inhibitors of Gene Expression: Therapeutic Applications" held on June 18-21, 1989 in Rockville, Maryland; and a 2-day workshop on "Regulatory Issues for Hematopoietic Growth Factors (HGF)" held on May 8-9, 1989 in Bethesda, Maryland.

INTRAMURAL PROGRAM ACCOMPLISHMENTS

The intramural basic and clinical research efforts are highly integrated. There are numerous collaborations between the intramural laboratories and the laboratories have a major impact on the clinical trials conducted both intramurally and extramurally. There is an annual BRMP scientific retreat at which the progress of individual projects is reviewed and discussed. There are regular meetings of the scientists and the clinicians called Therapeutic Applications Group meetings organized around particular areas of science from which clinical applications are developed. There is abundant scientific exchange in weekly staff meetings and monthly laboratory and branch chiefs meetings. There is an active seminar program at which intramural scientists and extramural scientists from all over the world come to discuss their most recent findings. In addition, BRMP scientists are frequent organizers, session chairmen, and presenters at national and international scientific meetings on basic and clinical science.

Office of the Associate Director

Research under the auspices of the Associate Director focusses on the development and control of proliferation of B cells and T cells. There are two tenured senior investigators working independently within the laboratories of the Associate Director, Drs. Jonathan Ashwell and Ada Kruisbeek.

Dr. Jonathan Ashwell has been studying the consequences of signal transduction through the T-cell receptor of thymocytes, normal adult T cells, and T-cell tumors. His work has demonstrated that the CD3 complex of the T-cell receptor exists in two forms in adult T cells, one comprised of a γ chain, δ chain, ϵ chain, plus a ζ-ζ homodimer (80% of the CD3 molecules); and the other comprised of a γ, δ, and ϵ chain, plus a ζ-η heterodimer (20% of the CD3 molecules). Analysis of mutants lacking the η chain have demonstrated that the CD3 molecules containing the ζ chain are responsible for phosphoinositide turnover as a consequence of signalling, whereas the ζ-ζ homodimer stimulates tyrosine kinase activity. Lymphokine production as a consequence of signal transduction is mediated by the ζ-ζ homodimer. T-cell tumors that die after stimulation with their antigen are capable of undergoing growth arrest in the absence of the

η chain, but do not autolyze. Thus, autolysis, which is characterized by the induction of an endonuclease that destroys the cell's DNA, requires phosphoinositide turnover and an intact η chain. The process of cell death is blocked by cyclosporin A and requires extracellular calcium ions. Dr. Ashwell noted the similarity between the activation-induced cell death in T-cell hybridomas and the process of negative selection of T cells in the thymus and he examined the composition of the CD3 complex in thymocytes. He found that the proportion of η chain-containing CD3 molecules was maximal early in ontogeny and declined sharply by birth. This sequence of events is consistent with the notion that negative selection in the thymus is a consequence of signalling through an η chain-containing CD3 molecule that leads to the death of the cell. Further experiments to address this possibility are being conducted. One practical consequence of this work has been the development of two clinical protocols using anti-CD3: (1) the use of anti-CD3 in CD3+ T cell tumors (it is hoped that the CD3 contains the η chain and that signalling through CD3 in T-cell tumors will result in their death); and (2) the use of anti-CD3 as a stimulant to T-cell immunity.

Dr. Ada Kruisbeek has performed pioneering in vivo studies demonstrating the requirements for the generation of the T-cell repertoire for antigen recognition which occurs in the thymus. She and her colleagues have shown that class II major histocompatibility complex (MHC)-restricted T cells, which are mainly the CD4+ helper cells, require signal transduction through the IL 2 receptor, through CD4, and need to encounter class II MHC antigens in the thymus in order to develop. If any of the three signals is blocked, the CD4+, class II MHC-restricted T-cell population fails to develop. Similarly, class I MHC-restricted, CD8+ T cells will fail to develop if either class I MHC or CD8 is blocked with antibody during T-cell development. She is now pursuing the cellular basis for these signalling requirements in an in vitro fetal organ culture system that largely recapitulates the in vivo thymus. In this system, she is evaluating the effects of IL 2 and IL 4 on T-cell development with specific monoclonal antibodies that antagonize the effects of these lymphokines.

In collaboration with Dr. James Kenny of Program Resources, Inc., the laboratory is also evaluating the development of B cells in a transgenic mouse system in which an intact immunoglobulin heavy chain and light chain gene has been inserted into the germline under the influence of the immunoglobulin promoter region. The resulting mice have a somewhat reduced complement of B cells 97% of which express the transgene product, an antibody that binds to phosphorylcholine. The other 3% of B cells express endogenous rearranged immunoglobulin genes and about 5% of the transgene positive B cells also express endogenous immunoglobulin genes, thus, the feedback inhibition of endogenous gene rearrangement is not quite complete. The transgenic B cells appear to respond to cross-linking of the surface Ig in a markedly reduced way compared to normal B cells. Efforts are underway to analyze the nature of signal transduction using the phosphorylcholine antigen and to evaluate what signals are required from T cells to augment the normal antibody response. In addition, the transgenic mice have been bred with animals expressing the xid defect, an X-linked immunodeficiency in mice predominantly affecting the response to T-independent antigens. The expression of the transgene did not correct the xid genetic defect and, in fact, such mice were profoundly B-cell deficient. An analysis of the cell populations of B cells and B-cell precursors in the marrow demonstrated a marked reduction of surface Ig-expressing cells but a normal number of B-cell precursors. This suggests that part of the defect in xid mice must be the abnormal clonal deletion of B cells expressing antibodies with the capacity to recognize T-independent antigen

specificities. A prediction of the tolerance model is that transgenic mice expressing an immunoglobulin molecule specific for a T-dependent antigen would not demonstrate the profound reduction in B-cells seen in phosphorylcholine transgenic mice. Such experiments have supported the notion that clonal deletion is limited to animals expressing antibodies recognizing T-independent antigens.

Drs. Margaret Beckwith and Chou-Chi Li of Program Resources, Inc. are collaborating with the Associate Director on a project to understand the mechanism of growth arrest of malignant B cells undergoing signal transduction through their surface immunoglobulin or other stimuli such as phorbol esters. Surface immunoglobulin positive tumors undergo irreversible growth arrest when stimulated with anti-Ig or phorbol esters. Not all of the growth arrest is reversible by antagonists of protein kinase C. In fact, it appears that one consequence of signal transduction is the induction of receptors for TGF-β, which is also produced by the cells. Thus, one contribution to the growth arrest may be an autocrine growth inhibitory effect of TGF-β. This implies that a mechanism of lymphomagenesis may be the loss of expression of TGF-β receptors and thus, the loss of normal negative growth control. Another interesting aspect of this work is that different signals can be used to produce growth arrest at distinct phases of the cell cycle. For example, anti-mu induces an arrest in G1 while dolostatin induces a G2/M block. Efforts are underway to sort out the mechanism of the block with particular emphasis on the regulation of the cell cycle control proteins such as cdc2, the most oncogene product, and cyclin.

Laboratory of Molecular Immunoregulation

The Laboratory of Molecular Immunoregulation (LMI) investigates at a molecular level the intercellular and intracellular process that regulate host defense mechanisms. This research is carried out by three investigators, Drs. Joost Oppenheim, Kouji Matsushima, and Scott Durum.

Dr. Joost Oppenheim is an internationally renowned immunologist whose recent work focusses on the use of IL 1 in vivo to protect against the lethal effects of radiation and chemotherapy. He has used IL 1 to enhance the survival of mice given up to 1500R (1.5x the LD100) of radiation followed by syngeneic or allogeneic bone marrow transplantation. In mice receiving allogeneic bone marrow, he has found that IL 1 is capable of preserving enough autologous marrow stem cells that animals develop mixed chimerism. Because the allogeneic cells are present during the recovery of the host marrow, the host hematopoietic and lymphoid cells develop tolerance to the allogeneic donor cells and immunologic competence to recognized third party allogeneic cells is retained. If such experiments can be replicated in a larger animal model (e.g. pig, primate), it may be possible to remarkably enhance the efficacy of the preparative regimen in bone marrow transplants and to make allogeneic transplantation considerably safer and less devastating to immune competence. As a direct result of Dr. Oppenheim's laboratory efforts with IL 1, the Clinical Research Branch is conducting a Phase Ia/Ib clinical trial of IL 1-α with the goal of following the first study with a study to evaluate the myeloprotective effect of IL 1 against radiation therapy and chemotherapy.

Dr. Kouji Matsushima has continued to be remarkably productive. He and his colleagues are analyzing the cellular consequences of signal transduction through the IL 1 receptor. He has found that the responses of cells to both IL 1 and tumor necrosis factor (TNF) have similar features including down regulation of

ornithine decarboxylase, induction of manganese superoxide dismutase, and the phosphorylation of two intracellular proteins, p65 and p74. The p65 substrate has been identified as l-plastin. Both proteins are phosphorylated on serine residues and the enzyme responsible for the phosphorylation is distinct from protein kinases A, protein kinase C, and casein kinase. Dr. Matsushima and his colleagues have made dramatic progress in their characterization of two IL 1 (and TNF)-inducible leukocyte chemotactic and activating factors. The best characterized has been called IL 8, which is a monocyte-derived neutrophil, lymphocyte, and monocyte chemotactic factor produced as a 99-amino acid precursor that is subsequently cleaved to 72 amino acids in length. IL 8 shows amino acid sequence homology with platelet factor 4, β-thromboglobulin, and other small molecular weight growth factors. The gene has been cloned and functional recombinant IL 8 produced in both E. coli and CHO cells. The gene for IL 8 maps to chromosome 4 q12-21. It induces the margination and emigration of neutrophils and its effects in vivo appear to mimic the capillary leak syndrome seen with high-dose IL 2 therapy in man. Receptors for IL 8 are distinct from other cytokine receptors. In addition to IL 8, this laboratory has identified a distinct monocyte chemotactic and activating factor that bears sequence homology with several new cytokines such as JE, LD 78, RANTES, TCA-3, and ACT-2. In vivo effects appear to be specific to monocytes. Both newly identified cytokines, IL 8 and MCAF, have enormous clinical potential in cancer and infectious diseases.

Dr. Scott Durum has been examining the roles of cytokines on thymocyte proliferation and the roles of IL 1 and antigen on mature T-cells. He and his colleagues have found that IL 1 induces the expression of the jun oncogene and antigen induces fos. With these two proteins induced by two distinct signals, jun and fos can interact and lead to the formation of the AP-1 transcription factor which is a DNA binding protein that activates the IL 2 promoter. Thus antigen and IL 1 are both necessary for the routine genetic mechanism of IL 2 induction in mature T cells. Durum and colleagues have also begun to analyze a subtraction library of IL 1-induced genes to assess the cellular consequences of IL 1 stimulation. The Durum laboratory is also interested in the cytokine regulation of thymocyte proliferation. They have found that IL 2 stimulates immature CD4- CD8- thymocytes to develop into mature CD8+ γ/δ T-cell receptor expressing T cells. On the other hand, the same population of immature thymocytes exposed to IL 1 generates CD4+ α/β T-cell receptor expressing T cells. This raises the possibility that IL 1 will be a useful way to expand CD4+ T cells in patients with AIDS.

Dr. Luigi Varesio is the head of the Immunobiology Section of the LMI. This section focusses on the cellular events responsible for the differentiation of lymphocytes and monocytes and their activation to exert antitumor effector functions. In a novel experimental system he and his colleagues developed for the study of macrophage biology, normal murine bone marrow cells are transformed with a recombinant retrovirus containing both v-raf and v-myc genes. Such cells form tumors in mice but are capable of being induced to differentiate into cells that behave like mature macrophages. Dr. Varesio has found that a combination of picolinic acid and interferon-γ is capable of inhibiting the proliferation of these retrovirus-transformed cells. These agents appear to destabilize the oncogene mRNA. Thus, the induction of post-transcriptional events can lead to tumor cell growth inhibition. Dr. Varesio and his colleagues have also examined the molecular events that lead to the induction of cytotoxicity in macrophages. IL 2 can directly activate human monocytes to become cytotoxic. These cells constitutively expressed the high-affinity IL 2 receptor and exposure to IL 2

results in the induction of c-fms, the M-CSF receptor, activation of protein kinase C and the development of cytotoxicity. M-CSF can potentiate the cytotoxicity of IL 2-induced monocytes. Interferon-γ can also induce monocytes to become cytotoxic however, it does not induce c-fms, does not stimulate protein kinase c and M-CSF has no augmenting effects on the interferon-γ-induced monocyte activation. Dr. Varesio's group has also demonstrated that macrophages latently infected with HIV constitutively express nuclear proteins that bind to the HIV-LTR. Activation of macrophages with lipopolysaccharide induces additional LTR binding proteins that can induce HIV expression. Regulation of expression of HIV in macrophages, a major reservoir of the virus in man, could lead to novel therapeutic strategies for AIDS.

Dr. Frank Ruscetti is the head of the Lymphokines Section which investigates the mechanism of action of lymphokines at the biochemical and molecular level. Dr. Ruscetti and his colleagues have discovered that TGF-β is a bifunctional regulator of hematopoiesis. They had previously shown that early bone marrow progenitors (and the leukemic cells derived from early progenitors) were exquisitely sensitive to the antiproliferative effects of TGF-β and that TGF-β1 and TGF-β2 were equipotent in their effects. On the other hand, more mature precursors did not express receptors for TGF-β and were insensitive to its growth inhibitory effects. In contrast to its growth inhibitory effects when used alone, addition of TGF-β together with GM-CSF greatly augmented marrow cell growth, especially within the granulocyte compartment. Bone marrow cells enriched for high proliferative potential stem cells by prior treatment with 5-fluorouracil are dependent on the combination of TGF-β plus GM-CSF for growth. The mechanism of these paradoxical results is under investigation.

Dr. Ruscetti is also examining the mechanism of viral latency in HTLV-I- and HIV-infected cells. HTLV-I-infected B cells possess integrated HTLV-I provirus but express no viral message or proteins unless they are stimulated with phorbol esters. Transfection of DNA under the influence of the HTLV-1 LTR or tax regions fails to get expressed in HTLV-I-infected B cells but is abundantly expressed in T cells. Thus, there appears to be a B-cell gene product capable of negatively regulating HTLV-I expression. Studies are underway to identify that protein and its mechanism of action. HIV latency is also a serious problem. Dr. Ruscetti has developed a model for HIV latent infection of human monocytes. He has found two forms of so-called latency: (1) low expression of viral transcription, and (2) no expression of virus. In the first form of latency (which is not really latency as usually defined), expression is regulated partially by monocyte nuclear proteins which can be down-regulated by monocyte activation signals, e.g. lipopolysaccharide. The second form of latency appears to be the result of methylation of the HIV-LTR since it is reversible by 5-azacytidine, but not by usual activation signals. It is thus, clear that host regulatory factors can affect HIV expression. We believe this is a potentially fruitful line of investigation that may lead to novel antiviral therapies.

Dr. William Farrar is the head of the newly established Cytokines Molecular Mechanisms Section and is one of our most successful scientists. The mission of this new section is to characterize common biochemical pathways shared by the immune system and the nervous system and to characterize the interaction of HIV with cells of the immune and nervous systems. Dr. Farrar's group is studying the nature of the signal transduction mediated by growth factors whose receptors do not have intrinsic kinase activity. They have found that many receptors that do not have their own intracellular domains, e.g. IL 2 receptors, IL 3 receptors,

and the receptors for other hematopoietic cytokines, stimulate tyrosine kinases to mediate their signal. Although there may be certain differences between the kinases, it appears that the program of gene expression that follows as a consequence of ligand-receptor interactions is much the same in IL 2-responsive cells responding to IL 2 and in IL 3- or GM-CSF-responsive cells responding to their ligands. Nuclear proto-oncogenes fos, myb, and myc are induced, ornithine decarboxylase is induced, and heat shock proteins are induced. Interference with myc expression either by use of cyclic AMP or antisense oligonucleotides to myc can block the proliferative response of T cells responding to their activation signals (e.g. phytohemagglutinin). Efforts are underway to identify the specific cellular proteins that are phosphorylated by growth signals and to understand their role in the events that lead to cell proliferation. In addition, Dr. Farrar and his colleagues are examining the DNA binding proteins that lead to particular patterns of gene expression in hematopoietic cells responding to growth factors.

Dr. Farrar has also been interested in understanding the relationship of HIV infection to the stimulation of cytokine release in infected cells. His group has shown that the gp120 HIV envelope protein is a potent stimulator of the release of monokines such as IL 1 and TNF from monocytes. In addition, gp120 interferes with leukocyte chemotaxis and function. He has also found that the cytokines released upon exposure to gp120 are capable of stimulating HIV viral expression in latently infected cells. Thus, his studies add significantly to our understanding of HIV effects on the immune system.

Laboratory of Experimental Immunology

The Laboratory of Experimental Immunology (LEI) conducts studies on biological response modification and the application of these studies to the treatment of cancer. Dr. John Ortaldo is the Chief of this laboratory.

The Leukocyte Differentiation Section has Dr. John Ortaldo as its acting head. The section studies the differentiation and activation of human and murine lymphocytes and studies the role of the cellular immune system in mediating antitumor immune responses. Dr. Ortaldo's group is making remarkable progress in its effort to understand the basis for cytotoxicity mediated by large granular lymphocytes (LGLs), which are the effectors of natural killer (NK) and lymphokine-activated killer (LAK) activity. In the course of studying the nature of the antigen recognized by NK cells through generating antibodies to an NK target cell (K562), they developed an antibody that blocked the binding and lysis of the target by LGLs. An anti-idiotype to that anti-target cell antibody could react with the NK antigen receptor. Such an anti-idiotype antibody is capable of blocking LGL cytolysis and identifies two proteins, and 80-100 kD and a 160 kD protein. With rabbit anti-idiotype (anti-NK receptor) serum, a gene has been cloned that appears to encode for the receptor. Sequence analysis demonstrates that this single copy gene does not have homology to any known genes. One of the granule proteins thought to be involved in cytolysis mediated by LGLs is called natural killer cell cytotoxic factor or NKCF. Its molecular weight is 12,000 and newly developed antibodies to this factor neutralize NK cell killing in vitro. It is anticipated that the antibodies may be useful in characterizing this NKCF further.

Dr. Ortaldo has been instrumental in demonstrating the potent immunoregulatory capacity of LGLs in immune responses. They produce a wide range of cytokines in

response to a single stimulus, IL 2. Since LGLs do not express the CD25 Tac antigen IL 2-receptor α chain, Dr. Ortaldo's group hypothesized that they constitutively express only the β chain of the IL 2 receptor and that IL 2's effects on LGLs were through this receptor. He has now demonstrated the accuracy of that hypothesis with antibodies to the p75 IL 2 receptor. He has begun to characterize the nature of signal transduction through the β chain of the IL 2 receptor including the effects on phosphorylation of protein substrates and the effects on gene expression.

Another interesting observation made by investigators in the Leukocyte Differentiation Section deals with the ontogeny of LGLs. They have shown that LGLs and mature T cells have a common progenitor, the dull Lyl+ thymocyte. Dull Lyl+ thymocytes can repopulate both the T cell compartment and the hepatic LGL population of a lethally irradiated mouse. Further work on the heterogeneity of LGLs in the mouse have led to the development of a monoclonal antibody that separates murine LGLs into precursors of LAK cells (NK1.1+/LGL-1-) and precursors of NK activity (NK1.1+/LGL-1+). These two subpopulations appear to be nonoverlapping.

This year Dr. Howard Young was recruited to be the new head of the Cellular and Molecular Immunology Section. This section uses molecular approaches to investigate the mechanisms by which the immune system can be augmented and the mechanisms by which tumor cell susceptibility to biological and immunological defense mechanisms can be enhanced. One of Dr. Young's major focus is to understand the mechanism of regulation of interferon-γ gene expression. He has found that there appears to be at least two enhancer-like regions in the human interferon-γ genomic DNA, at least one of which is tissue specific. Dr. Young has made transgenic mice expressing the human interferon-γ gene regulated in a physiologic way and intends to further study the in vivo regulation of gene expression.

This section is also studying a variety of other physiological processes. Using antibodies to the Pi isoenzyme of glutathione-S-transferase (GST-π), the role of this enzyme in drug resistance is being explored. An interesting byproduct of this work was the discovery that papillomavirus-infected cells, for example, cervical carcinoma cells, express GST-π in nearly 100% of cases. The antibody to GST-π is now undergoing investigation as a potential diagnostic reagent that could replace PAP smears with an automated technique. It's expression in colon cancer is also being evaluated. Using antibodies to the fos oncogene product, it has been shown that ras transformed cells constitutively produce a large amount of fos-related protein, however, mRNA levels for fos were quite low. The fos-related proteins are being isolated and characterized. Their inducibility and nuclear location suggests that they may be related to fos in function as well as structure.

Dr. Robert H. Wiltrout is the head of the Experimental Therapeutics Section of the LEI. This section strives to determine the antitumor efficacy and mechanisms of action of biological agents effective in the treatment of animal tumors in vivo. The laboratory has done extensive work to evaluate the basis of the remarkable synergistic antitumor effects mediated by the combination of flavone-8-acetic acid (FAA) plus IL 2. In collaboration with Dr. Howard Young, these workers have demonstrated that effective doses of FAA stimulate cytokine production including interferon-α, interferon-γ, and TNF. It may be that the synergy seen between FAA and IL 2 is related to the activity of one or more of the FAA-induced cytokines. Studies to define this synergy continue. The

laboratory is also attempting to develop therapeutic strategies specifically aimed at altering the expression of selected oncogenes in tumors that have been induced by oncogene-containing retroviruses. They have studied the macrophage cell line induced by transformation with v-raf and v-myc oncogenes, noted above that was generated by Dr. Luigi Varesio and his colleagues. They have found that interferon-γ and lipopolysaccharide partially inhibit growth, but the combination is synergistically effective and synergistically affects oncogene expression. Lipopolysaccharide induces the production of IL 1 and TNF by the tumor cells. Efforts are being made to replace the lipopolysaccharide with a recombinant cytokine in this in vivo system. Furthermore, efforts are underway to employ antisense oligodeoxynucleotides as therapeutic agents for this oncogene-related tumor.

Dr. Wiltrout's laboratory is also conducting in vivo experiments with TGF-β based on the in vitro experiments of Dr. Frank Ruscetti and his colleagues. They have demonstrated that regional administration of TGF-β can transiently arrest the proliferation of early hematopoietic stem cells. Furthermore, like IL 1, TGF-β can protect the bone marrow against the toxic effects of a lethal dose of cyclophosphamide. Further studies are aimed at evaluating combinations of myeloprotective and myelorestorative agents and optimizing the dose-intensity of chemotherapeutic agents.

Another major emphasis of Dr. Wiltrout's laboratory is understanding in vivo effects on the trafficking of cells of host defense. He and his colleagues have demonstrated that certain biological response modifiers are capable of augmenting organ-associated NK activity. This increase is mediated not by mobilizing circulating NK cells or splenic NK cells. The increase is derived from an increase in the generation of NK cells in the bone marrow. Further experiments are designed to detect the factors responsible for the generation of the cells in the marrow. Together with his colleague, Dr. Thomas Sayers of Program Resources, Inc., Dr. Wiltrout is evaluating the in vivo migration of host defense cells. IL 1 has been found to be a potent stimulus for inducing neutrophil migration into the peritoneal cavity. IL 1 is more potent than TNF but suboptimal amounts of IL 1 can be augmented by TNF. NK cells did not increase their trafficking into the peritoneal cavity in response to IL 1 and/or TNF but did chemotax in response to IL 2 and interferon-γ. The IL 2 response was blocked by antibody to interferon-γ, thus, the effects of IL 2 on NK cell migration are indirect. In vitro, interferon-γ does not appear to be capable of inducing NK cell chemotaxis across a membrane, thus, it appears most likely that interferon-γ is inducing a distinct chemotactic factor to account for the results. Understanding how to get host defense cells where we want them to go is critical to the development of effective therapies. Dr. Anne Pilaro has demonstrated that some host defense cells bear laminin receptors and fibronectin receptors and use them to bind to intercellular matrix proteins in vitro. Such receptors are likely to be involved in the migration of such cells to sites of inflammation.

Dr. John Pearson is conducting in vivo studies aimed at rationally combining chemotherapy and immunotoxin therapy. He has shown in human tumor xenograft models of colon and ovarian cancer that combination chemotherapy with immunotoxins can produce 5-log reductions in tumor burden in vivo. He is also exploring methods of enhancing the therapeutic efficacy with strategies aimed at augmenting the cytotoxicity of immunotoxins, including the use of calmodulin inhibitors and combinations of immunotoxins internalized into the tumor cell via distinct pathways.

Laboratory of Biochemical Physiology

The Laboratory of Biochemical Physiology under the direction of Dr. Hsiang-fu Kung is charged with performing studies on the genetic and biochemical events related to the development and expression of the malignant phenotype. Dr. Kung and his colleagues are elaborating the mechanisms of action of ras oncogenes. The ras gene family codes for a membrane-associated protein called p21 that is closely related to the G-protein family of proteins involved in growth signal transduction. Dr. Kung's group has recently purified and cloned the ras GTPase activating protein (GAP) and studied its function by micro-injection into Xenopus oocytes. GAP induced a rapid increase in diacylglycerol but no important increase in phosphoinositide turnover. Thus, the source of the diacylglycerol appears to be a phospholipid other than phosphoinositides. Similar results are obtained with the injection of T24 ras protein. Injection of ras protein induced oocyte maturation, but GAP did not, thus, an increase of diacylglycerol formation alone is not sufficient for the induction of oocyte maturation. Some additional signal delivered by ras must be involved. Dr. Kung and his colleagues are also examining the mechanism of action of cellular proteins that appear to suppress the functions of the ras protein, including two small G proteins (smg or rap, and ADP-ribosylation factor or ARF). Such studies may lead to treatment strategies aimed at regulating ras expression, which has been implicated in over 90% of pancreatic cancers and the majority of colon cancers.

Dr. Kung has also studied the role of phospholipase C in cellular transformation. Microinjection of phospholipase C into quiescent NIH 3T3 cells led to induction of DNA synthesis and morphologic transformation. Co-injection of antibody to phospholipase C plus either phospholipase C or ras protein blocks induction of DNA synthesis. In contrast, antibody to the ras protein blocks induction of DNA synthesis by ras protein but not by phospholipase C. Thus, it appears that phospholipase C activity is downstream of ras in the signal transduction pathway.

Dr. Kung and his colleagues have also generated data that help understand the activities of certain cytokines. For example, together with Drs. Mark Smith and Scott Durum, they have shown that TNF microinjected into TNF-sensitive L929 cells induces a calcium-dependent endonuclease that breaks down the DNA of the cell. TNF-resistant L929 cells are also resistant to TNF after injection and such cells are not induced to express the nuclease. Thus, the cytotoxic effects of TNF correlate with its capacity to induce a suicide enzyme that destroys DNA. Together with his colleague, Dr. Connie Faltynek of Program Resources, Inc., the laboratory is examining the biology of interferon-α receptors and IL 6 receptors. It appears that there are at least two forms of interferon-α receptors, one 90 kD and one 105 kD, with distinct affinities for the ligand (the 105 kD protein has a higher affinity) and distinct patterns of down-regulation in response to interferon. Drs. Faltynek and Kung have also examined the expression of IL 6 receptors on cells of a variety of lineages. Although the mRNA for IL 6 receptor is similar on all cell types examined, the cell surface receptor is significantly different in B cells, monocytes, and LGLs. Tissue-specific post-translational modifications of the receptor may thus be responsible for the distinct biological effects mediated by IL 6 in diverse cell lineages.

Dr. Faltynek has also studied the capacity of certain cytokines to induce hematopoietic tumor cells to differentiate in vitro. TNF and TGF-β are synergistic in their effects to induce monocytic differentiation in HL-60 and U937 tumor cells. Neither is effective alone. It appears that the initial

resistance of the cells to TGF-β is related to the absence of receptors, however, TNF induces the receptors. The combination of TNF and TGF-β will be explored in an animal model of HL-60-bearing nude mice.

Dr. Kung's laboratory has a number of approaches to understanding AIDS and developing new therapeutic strategies. They have found that interferon-γ can down-regulate the expression of CD4 on monocytes and therefore may help block this pathway of monocyte infection by HIV. They are also collaborating with Dr. Frank Ruscetti in determining the nature of the negative regulatory elements (DNA binding proteins) present in HIV-infected monocytes. Modulation of HIV transcription is another novel therapeutic strategy. Finally, Dr. Kung and his colleagues have demonstrated that the use of chloroquine in vitro significantly alters the glycosylation of the gp120 envelope protein and results in the production of virions that are substantially less infectious. Thus, it is possible that this or other inhibitors of glycosylation could result in a therapeutic effect not only by reducing infectivity but by altering the gp120 effects on host defense.

AIDS-Related Research in BRMP Intramural Laboratories

Of course, it is our hope that much of the information we obtain in the course of studying normal immune physiology will prove useful in fighting AIDS and its many manifestations. However, in addition, BRMP laboratories are engaged in a number of specific research projects that have AIDS as their primary focus. The BRMP AIDS research effort has three major components: 1) to understand the regulation of virus production and viral pathophysiology; 2) to attack the virus by a molecular or immunological strategy; and 3) to reconstitute the immune system after viral replication is controlled.

1. To understand the regulation of virus production and viral pathophysiology

The BRMP is studying the genetic mechanisms that regulate viral replication in T cells and monocytes and has discovered that viral latency occurs by distinct mechanisms in the different cell types. In T cells, viral latency appears to be related to host cell DNA binding proteins that recognize the viral LTR and block expression. Unfortunately, the pathway of T cell stimulation results in the induction of DNA binding proteins that appear to compete with those binding to the HIV LTR in resting T cells. Since the LTR contains motifs similar to a variety of lymphokines produced by stimulated T cells, such stimulatory NF-kappaB type DNA binding proteins activate latent HIV expression when the T cell is activated. However, it may be possible to alter this scenario and efforts are underway to identify the repressor DNA binding proteins. In monocytes, the virus has two forms of latency. In one form, virions are made but not extruded from the cell. These monocytes are only infectious when they are damaged and virions leak out of the cell. The second form of latency appears to involve methylation of the HIV viral LTR. It is not yet known what controls the susceptibility of the LTR to DNA methylation, however, this may provide yet another therapeutic target.

Efforts are also underway to understand how HIV produces the changes it makes in immune function. For example, it is now clear that viral components can have direct trophic effects on the cells of the immune system and the nervous system and some of these may be related to certain disease manifestations, for example, the autoimmune thrombocytopenia or the neurologic changes. Some of the organ effects in AIDS resemble autodestructive lesions mediated by the immune system

rather than the effects of immunodeficiency. Thus, our studies on mechanisms of self-tolerance and suppression may relate to therapeutic strategies for AIDS. Finally, insights into the pathophysiology of AIDS are being sought by examining lymphoid and hematopoietic cell development and function in a murine AIDS model.

2. To attack the virus by a molecular or immunologic strategy

The BRMP is exploring a number of novel approaches to attack the virus that might complement the antiviral agents that have been demonstrated to affect HIV viral replication such as AZT and other dideoxynuclosides. For example, certain lymphokines appear capable of significantly reducing the expression of CD4 molecules on the surface of cells that are targeted by the virus. Some biological signals can alter viral replication. HIV envelope analogs may interfere with HIV binding to its target. Certain peptides have been suggested to interfere with viral infectivity without binding to CD4 or competing with gp120 binding. Sorting out the cellular mechanism of these inhibitors may result in a new treatment strategy.

It is clear that some patients with HIV infection have some serum antibody that successfully blocks virus infection and BRMP scientists have demonstrated that NK and LAK cells are very potent at killing HIV-infected cells. Thus, strategies to boost host immunity to the virus may be clinically useful.

Finally, the evidence from monocytes that are latently infected with HIV is that viral production may depend crucially on DNA binding proteins, DNA methylation, or protein glycosylation pathways in the host cell and particular intracellular compartments. Such dependencies may create vulnerabilities that can be attacked to limit HIV replication and production.

3. To reconstitute the immune system after viral replication is controlled

The BRMP is examining thymus and bone marrow transplantation as strategies to reconstitute T-cell immunity. Furthermore, it appears that the use of certain cytokines, for example IL 1, may facilitate the regeneration of CD4+ T cells that are severely compromised in AIDS patients. Once the enormous problem of viral replication is successfully addressed, we shall still be left with the enormous problem of giving successfully treated patients a new immune system.

The Clinical Research Branch

The Clinical Research Branch (CRB) has Dr. Dan L. Longo as its acting chief. For the first time in the last 5 years, the Clinical Research Branch is now fully staffed with 5 senior investigators. The CRB is responsible for the clinical testing of biological therapies, their integration with other modalities of cancer treatment, and the correlation of in vitro parameters of immune and biological function with in vivo antitumor effects. The CRB consists of a 13-bed inpatient unit that includes a 4-bed intensive monitoring, and an additional 3-bed cytapheresis unit located in the Frederick Memorial Hospital. In January, 1989, the outpatient unit moved to a brand new facility located in the Frederick Memorial Hospital Cancer Treatment Center located across the street from the Hospital.

The clinical activities of the CRB are diverse. The clinical effort depends heavily on extremely close collaboration with Dr. Walter Urba and his colleagues in the Clinical Immunology Laboratory of Program Resources, Inc., perhaps the

finest clinical monitoring laboratory in the world. Currently there are active protocols testing recombinant cytokines alone and in combination, recombinant cytokines with adoptive cellular therapies, recombinant cytokines with single agent chemotherapy and combination chemotherapy, recombinant cytokines with classic biological response modifiers, recombinant cytokines with adoptive cellular therapy plus chemotherapy, monoclonal antibody therapy, immunotoxin therapy, and a number of other treatment strategies.

This year the CRB published its results with the use of intraperitoneal LAK cells plus IL 2. The response rate was 35% in patients with ovarian cancer or colon cancer limited to the peritoneal cavity. There was an enormous pharmacological advantage to administering the cells intraperitoneally. Ascites levels of IL 2 were 10- to 100-fold higher than serum levels. In addition, interferon-γ was induced and there was evidence of in situ monocyte activation by the IL 2 or the cytokines it induced. The dose-limiting toxicity was the development of adhesions that prohibited further delivery of therapy. The toxicities associated with intraperitoneal IL 2 plus LAK cell therapy were qualitatively similar to those obtained with systemic LAK plus IL 2 therapy but were significantly less severe. Strategies to limit the development of adhesions are under consideration for a follow-up protocol.

The CRB is conducting a clinical trial combining systemic LAK plus IL 2 with combination chemotherapy using cyclophosphamide and doxorubicin followed by interferon-α in patients with renal cell carcinoma and melanoma. The response rate appears to be significantly higher than that we obtained with previous systemic LAK plus IL 2 regimens but further improvements are needed. We are about to begin a study in melanoma combining LAK plus IL 2 with the monoclonal antibody R24, an anti-GD3 antibody with a response rate of about 20% or so when used alone. LAK cells are capable of mediating antibody-dependent cellular cytotoxicity, thus, we are optimistic that the addition of an active antibody to LAK cells plus IL 2 will result in an incremental increase in response rates.

There are a number of ongoing studies that are evaluating the use of IL 2 chronically administered in a fashion to optimize the generation of LAK activity in the peripheral blood. Initial studies conducted with IL 2 seemed to indicate that responses were significantly more likely if the IL 2 were given in a dose and schedule that generated detectable levels of LAK activity in the peripheral blood. We are currently exploring the use of IL 2 together in four separate protocols each of which combines IL 2 with another immunomodulating agent. In one study IL 2 is given with cyclophosphamide. In another, IL 2 is given with poly-ICLC. Poly-ICLC is said to be a potent inducer of endogenous interferon-α and a number of laboratory studies have demonstrated that interferon-α plus IL 2 can produce synergistic antitumor effects. We also have a protocol employing IL 2 plus interferon-α. Finally, we are attempting to reproduce the dramatic synergy between FAA and IL 2 that was demonstrated in animal tumors by Dr. Robert Wiltrout. These studies are all actively accruing patients and are too early in their courses to make meaningful estimates of their outcomes.

Last year we published the first demonstration of an immunologically active dose of interferon-γ. We are now testing the antitumor effects of a chronically administered immunologically active dose of interferon-γ in patients with metastatic malignant melanoma.

There is substantial interest in the CRB in devising treatment protocols that will augment the dose-intensity of active chemotherapy programs. We have been studying the use of carboplatin plus GM-CSF in patients with relapsed ovarian cancer. Carboplatin is active in ovarian cancer but its dose-limiting toxicity is myelosuppression. In our study, we are trying to use GM-CSF to ameliorate the marrow toxicity. We have been able to administer high doses of carboplatin for 2, or at most 3 cycles with the support of GM-CSF and have seen responses, though our experience is not yet large enough to precisely determine the response rate. Unfortunately, many responding patients are unable to receive further doses of carboplatin because of the development of thrombocytopenia. GM-CSF appears to ameliorate the granulocytopenia but exerts no clinically discernible influence on thrombopoiesis. Thus, further exploration of dose-intense carboplatin awaits the development of regimens capable of protecting or restoring platelet counts. Certain laboratory experiments suggest that IL 1 may be capable of protecting animals against the myelotoxicity of carboplatin. Our ongoing Phase I study of IL 1 in man may set the stage for returning to dose-intense carboplatin as a salvage therapy in ovarian cancer.

Another effort to use CSFs to augment the dose-intensity of combination chemotherapy is being studied in patients with previously untreated poor prognosis advanced stage Hodgkin's disease. Our 25 years of experience with MOPP has provided us a substantial data base on the dose-intensity that can be delivered without bone marrow stimulants. We are attempting to augment the dose-intensity by using GM-CSF. The first 3 patients appear to be receiving an increased dose-intensity of MOPP, especially in the first 3 cycles, however, GM-CSF is associated with side effects such as fever, rash, fatigue, and myalgias that often results in mid-cycle hospitalization and prophylactic antibiotic administration. We are eager to enter a sufficient number of patients on the study to make valid statistical comparisons to our historical control group.

There are at least two ways to optimize dose-intensity. Give larger doses of therapy on the usual schedule or give smaller doses of therapy more frequently. In advanced stage Hodgkin's disease, we are taking the former approach and are using GM-CSF to stimulate the marrow. In advanced stage diffuse aggressive lymphomas, we are taking the latter approach with a regimen called short-course ProMACE-CytaBOM. Our previous ProMACE-CytaBOM regimen achieved a complete response rate of 84% with 67% of patients apparently cured of disease. We have devised a novel schedule using the same drugs but administering therapy on a weekly basis for 12 weeks instead of in 3-week cycles. Initial response rates appear quite high with short-course ProMACE-CytaBOM, but it remains too early to make any conclusions about its capacity to augment actual dose-intensity or about its response rate.

GM-CSF is not only a marrow stimulant, it is also a biological response modifier. When injected into the peritoneal cavities of mice, it called forth a potent influx of inflammatory cells into the peritoneal cavity. We are interested in this activity of GM-CSF in patients with malignancies limited to the peritoneal cavity. We are administering GM-CSF alone initially and then measuring the cytotoxic activity of the elicited cells. If the cells are not cytotoxic or are only weakly cytotoxic, we shall add either IL 2 or interferon-γ to augment their cytotoxicity.

The CRB is interested in targeted therapy. We have studied the administration of an immunotoxin called OVB-3-PE (generated by Dr. Ira Pastan and his colleagues) delivered intraperitoneally in patients with ovarian cancer. Although some minor

antitumor activity has been observed, the treatment is limited by the development of antibodies to both the toxin and the murine antitumor antibody. We have previously shown that deoxycoformycin, which is an effective therapy for hairy cell leukemia, induces significant alterations in T-cell function that might be useful in preventing the development of human anti-mouse antibody and human antitoxin responses. Other strategies include using an IL 2-diphtheria toxin conjugate that should only interfere with T-cells that are making an immune response and thus, selectively prevent the development of antibodies against the therapeutic immunoconjugate. Finally, there is some evidence in murine systems that administering an antigen together with autologous monocytes that have been exposed to cross-linking agents causes the antigen to be presented in a fashion that induces clonal anergy and long-lived tolerance. All three of these strategies to permit the more chronic administration of antitumor antibodies and immunotoxins are slated for testing in man.

The CRB is also testing some of the predictions generated by Dr. Jonathan Ashwell's work (vide supra). Cross-linking the T-cell antigen receptor (CD3) on malignant T-cell tumors should result in the delivery of growth inhibitory signals. We are examining the effects of anti-CD3 in T-cell tumors that express the T-cell antigen receptor. Furthermore, normal T cells exposed to anti-CD3 at doses that lead to cross-linking and activation rather than down-modulation of the receptor could be stimulated by anti-CD3. We are currently evaluating anti-CD3 as a T-cell stimulant in vivo and are designing a protocol to use anti-CD3 plus IL 2 in vitro to expand T cells with LAK activity for an adoptive cellular therapy protocol.

The CRB has recently purchased a gamma camera that will greatly enhance our ability to monitor the therapies we deliver. We will be able to do monoclonal antibody imaging and to label adoptively transferred cells to follow their trafficking. A number of imaging protocols are under development. It is our view that a major current limitation of biological therapy is our inability to deliver the therapy in adequate amounts to the tumor. We should now be able to explore a variety of maneuvers to improve delivery.

The approval of interferon-α for the treatment of patients with hairy cell leukemia has diminished our accrual of such patients for studies. However, we have made interesting observations on the treatment of this disease. First, our continuous administration of interferon-α to 34 patients for a median of 3-1/2 years is a novel treatment strategy. Most other series of patients have been treated for only 12-18 months. The other studies have found that patients relapse a median of 10-14 months after discontinuing interferon therapy and, unfortunately, not all of the patients respond to a second course of interferon. We have found that all of our patients are continuing to respond to chronic interferon-α therapy and we have evidence that some of the patients are continuing to improve through many years, as manifested by continuing improvement in peripheral blood counts and continued fall in serum soluble IL 2 receptor levels. The toxicities associated with chronic administration of low dose interferon are negligible. It may be best not to stop therapy at an artificially determined time point but to continue therapy as long as the patients continue to respond.

We have performed a study of alternating cycles of deoxycoformycin and interferon-α in 15 patients with hairy cell leukemia. Patients were treated for a total of 14 months and then treatment was stopped. All of the patients responded to the therapy and because of persistent rare hairy cells present in

the bone marrow, all were considered partial responders. However, all patients have remained stable in partial remission for periods up to three years off all therapy. Their counts have remained normal, their marrow hairy cells have not increased in number, and their serum levels of soluble IL 2 receptor, which we have previous demonstrated to be an excellent tumor marker in hairy cell leukemia, have remained normal. These patients have persistent immunologic defects related to the deoxycoformycin however, they do not appear to be prone to opportunistic infections. We are continuing to follow these patients for any untoward events.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09290-04 OAD

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antigen Presentation and T-Cell Activation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	J. D. Ashwell	Senior Investigator	OAD, NCI
Others:	M. Mercep	Visiting Fellow	OAD, NCI
	C. Zacharchuk	Medical Staff Fellow	MB, COP, NCI
	S. Volarevic	Visiting Fellow	OAD, NCI
	C. Burns	Visiting Fellow	OAD, NCI

COOPERATING UNITS *(if any)*
OAD, BRMP (J. Zuniga-Pflucker, A. Kruisbeek); Lab. of Cell Biol. and Metabolism, NICHHD, Bethesda, MD (A. Weissman and R. Klausner); Univ. of Minnesota, Rochester, MN (M. Jenkins); and Medical Biology Institute, La Jolla, CA (D. Ucker)

LAB/BRANCH
Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Activation of transformed T cells results in lymphokine production. Another major consequence is the inhibition of spontaneous growth, followed by cell lysis. The lytic response requires that extracellular Ca2+ be present and is prevented by co-culturing with cyclosporine A. Interestingly, the cell lysis is accompanied by the activation of endonucleases and the autolysis of nuclear DNA, manifested by DNA fragmentation. Activation of T cell mutants that do not express the T cell receptor (TCR) η chain (i.e., bear only the form of the TCR that contains the $\zeta 2$ homodimer) resulted in lymphokine secretion and a G1/S cell cycle block, but not cell death. Noting the similarity of activation-induced cell death and the thymic process known as negative selection, the expression of the TCR ζ and η chains during T cell ontogeny was examined. Both ζ and η were expressed early in the developing thymus, but declined sharply by birth. Furthermore, there was a disproportionately large decrease in η expression over this time interval, resulting in a decrease in the ζ:η ratio. Together, these data suggest that activation-induced cell death of T cell hybridomas is a legitimate model for the physiologic process of negative selection, in which the TCR ζ and η chains may play a crucial role. It should be possible to test this model by looking for functional correlations between ζ and η expression and biological responses in normal thymocytes and mature T cells.

PHS 6040 (Rev 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Investigator	OAD, NCI
Mladen Mercep	Visiting Fellow	OAD, NCI
Charles Zacharchuk	Medical Staff Fellow	MB, COP, NCI
Sinisa Volarevic	Visiting Fellow	OAD, NCI
Christopher Burns	Visiting Fellow	OAD, NCI

OBJECTIVES

(1) To study the mechanisms by which activation results in cell cycle block and growth inhibition of transformed T cells;

(2) To develop reagents that will act as a surrogate for antigen in the activation of a wide variety of lymphocytic neoplasms; and

(3) To understand what signals provided by APC are required for the successful presentation of antigen to T cells, especially with regard to the deleterious effects that gamma radiation and oxidizing agents have on this function.

MAJOR FINDINGS

I. Activation-Associated Growth Inhibition of Transformed T Cells

We have shown that normally mitogenic agents, such as the appropriate combination of antigen and MHC-encoded class II glycoproteins, antibodies to the T cell receptor (TCR), Thy-1 or Ly-6, and lectins, inhibit the growth of transformed T cells while they induce the production of lymphokines. Our studies have established that the growth inhibition is composed of two discrete steps. The first, a G_1/S cell cycle block, occurs within minutes of activation, does not require extracellular Ca^{2+}, and is unaffected by cyclosporine A. The second phase, cell death, is first evident 4 to 6 hours after activation, can be detected by the uptake of trypan blue or the release of ^{51}Cr, requires extracellar Ca^{2+}, and is prevented by cyclosporin A. These events are not mediated by lymphokines, but are the direct consequence of activation. We have continued to study the mechanism underlying "activation-induced cell death", bearing in mind the striking similarity between this phenomenon and the death of immature thymocytes that is induced by anti-TCR antibodies or steroids. We have found that activated T cell hybridomas, just as activated thymoctyes, lyse their own nuclear DNA. This event occurs within 4 to 6 hours after activation, the same kinetics with which cell death is detected by ^{51}Cr release. The fragmentation is inhibitable by zinc, implying that it is due to the activation of endonucleases. In addition, reagents such as Actinomycin D and cycloheximide prevent the DNA fragmentation, demonstrating a requirement for de novo transcription and translation. Cells bearing abnormal cell surface TCRs have yielded further information about activation-induced cell death. One series of T cell hybridoma mutants that fail to express $\alpha\beta$ in association with the $\zeta\eta$ heterodimer, but do express it with the ζ_2 homodimer, respond to antigen with the production of lymphokines and the inhibition of cell growth. However, antigen

fails to induce the lysis of these cells. The bulk of the evidence strongly supports the hypothesis that the in vitro phenomenon of activation-induced T cell death is analagous to the killing of thymocytes during negative selection. Furthermore, an implication of these studies is that the "death signal" is transduced by $\zeta\eta$-, but not ζ_2-, containing TCRs.

II. The TCR in Thymic Ontogeny

Because of the apparent relationship between TCR $\zeta\eta$ heterodimer expression and susceptibility to activation-induced cell death, studies have been performed to determine how this TCR subunit is expressed in the thymus. Highly purified thymocyte populations were prepared in the laboratory of Dr. Ada Kruisbeek. Detection of three of the TCR subunits (α, ζ, and η) has been performed by immunoblotting with specific antibodies followed by autoradiography. In the unfractionated adult thymus, all three chains are detectable. Of special interest is the ζ:η ratio, which in peripheral T cells is approximately 20:1. In adult thymus, the ratio is 2- to 4-fold lower. All of the cellular fractions from adult thymus that we have examined express detectable ζ and η, with the hierarchy (greatest to least) being $CD4^+$ and $CD8^+$ > $CD4^+8^+$ > $CD4^-8^-$. Since the adult thymus represents a steady-state population with many cells undergoing differentiation in an unsynchronized fashion, we have also studied TCR $\zeta\eta$ expression during fetal ontogeny. ζ and η expression is first readily detectable on day 17 of development. The levels of both chains (with a ζ:η ratio of about 10:1) remains stable for several days and then both rapidly decline. By the day of birth (a day 20 to 21 of fetal development) ζ levels are decreased 5- to 10-fold. More impressively, η levels are virtually undetectable, implying a change in the ζ:η ratio as well as in the absolute amount of both chains. The results have been confirmed in fetal organ culture, although kinetic studies have suggested a delay of 1 to 2 days compared to the intact animal. In contrast to ζ and η, the α chain is found at relatively low levels on day 17, and reaches a plateau on day 18 that extends through the first week after birth. Also, it is important to note that the level of cell surface TCR expression (detected by immunofluorescence staining) is unchanged from day 17 to 21 of fetal development. These data demonstrate that the TCR ζ and η chains are regulated during ontogeny independently of α, and independently of total cell surface TCR expression. Studies are currently underway to investigate the significance of this in terms of T cell selection.

III. Antigen Presentation by Resting B Cells

Resting B cells can present soluble antigen to T cells. However, their potency (that is, the amount of antigen they require to stimulate T cell responses) is generally 10- to 30-fold less than "professional" antigen-presenting cells such as dendritic cells. Many studies have suggested that this difference is due to the B cells inability to effectively express antigen/Ia molecule complexes on the cell surface. This could be due to, for example, low Ia molecule levels, relative inability to take up and process antigen, or failure to express adhesion molecules that help to increase the avidity of the TCR for its ligand. An alternative model that we have proposed is that B cells are relatively deficient in poorly-understood "costimulatory" signals that normal T cells (but not T cell hybridomas) require for optimal IL-2 and proliferative responses. In collaboration with Dr. Marc Jenkins we have directly tested these alternate

(although not mutually exclusive) models by using antigen-driven T cell phosphatidylinositol (PI) hydrolysis as a measure of TCR occupancy. For the T cell clones employed in these studies we have found PI hydrolysis to occur within minutes of activation and to be sustained at plateau levels from 1 to 5 hours after stimulation. Direct comparison of T cell proliferation or IL-2 production when resting B cells vs. dendritic cells were used as APC found that the latter were approximately 1000-fold more potent. In contrast, using PI hydrolysis as a read-out, the latter were only 6-fold more potent when PI hydrolysis was measured 1 hour after activation, and no more potent when this measurement was done 3 hours after activation. In contrast, the "add back" of costimulatory activity by the addition of allogeneic dendritic cells that cannot present the antigen to the T cell clones, largely restored the ability to these clones to produce IL-2 and proliferate, without enhancing their hydrolysis of PI. These results strongly indicate that resting B cells are similar to dendritic cells in their ability to take up, process, and present antigen to normal T cell clones. Their deficiency is in the provision of secondary, TCR-independent, costimulatory signals to the T cell.

IV. Physical Associations Between Biologically Active T-Cell Molecules

Multiple independent T cell surface molecules (e.g., CD2, Thy-1, and Ly-6) transduce activation signals. A paradox is that Thy-1 and Ly-6 are not transmembrane proteins, but are linked to the plasma membrane via a phosphatidylinositol anchor. One clue to the mechanism by which these molecules signal is that some, but not all, of the activation events they mediate require co-expression of the TCR on the T cell surface. We have hypothesized that these molecules physically interact, directly, or indirectly, with the TCR. To test this, we have treated functional T cells with homobifunctional cross-linking reagents and then immunoprecipitated with antibodies to molecules of interest. Initial studies with a T-cell hybridoma revealed that Thy-1 could be cross-linked to at least four major independent cell surface molecules, with molecular weights of 180,000, 70,000, 42,000, and 12,000. All of these bands, with the exception of the 70,000 band, were also seen using purified splenic T cells. Therefore, we have tentatively identified this band as the viral gp70 glycoprotein. The other bands have been identified by either immunoblotting or cross-linking and immunoprecipitation from specific T cell loss mutants. The 42,000 and 12,000 bands represent MHC-encoded class I molecules and β_2-microglobulin, respectively. The 180,000 band is CD45 (also known as T200 and common leukocyte antigen). The latter was of especial interest because it has recently been shown that phosphorylation of intracellular substrates on tyrosine is an important pathway in T cell activation. Strikingly, immunoprecipitation from cross-linked cells with antibodies to the TCR, but not to the transferrin receptor or the adhesion molecule LFA-1, also brought down CD45. To determine the potential functional significance of this obervation, assays of tyrosine phosphatase activity have been performed on immunoprecipitates from cross-linked and uncross-linked T cells. Preliminary studies indicate that from cross-linked membranes only, anti-Thy-1, and CD3-ϵ, and anti-TCR-α monoclonal antibodies bring down tyrosine phosphatase activity; antibodies to the transferrin receptor do not. These studies prove that Thy-1 and the TCR independently interact with a third, transmembrane protein, CD45, that has demonstrated enzymatic activity. Our current hypothesis is that this interaction underlies the ability of Thy-1 to signal, and accounts for the dependence of some signals on co-expression of the

TCR. Future experiments will seek to directly test this model in T cell mutants that have the TCR and Thy-1, but have lost CD45 expression.

SIGNIFICANCE

The continuing study of the in vitro phenomenon of "activation-associated growth inhibition" of transformed T cells has suggested that it has relevance to the development of normal T cells. In particular, activation of both T-cell hybridomas and normal developing thymocytes undergoing negative selection results in cell death accompanied by DNA fragmentation. In both cases the death is antigen-specific, requires extracellular Ca^{2+}, and is sensitive to CsA. Of especial interest is why thymic T cells die when they encounter antigen while normal T cells proliferate. The work cited above suggests that the answer may lie in the composition of the TCR. That is, signalling through receptors bearing the $\zeta\eta$ heterodimer preferentially causes cell death. Indirect evidence for this comes from the finding that these two chains are highly regulated during ontogeny. Further experimentation on the biological responses of fetal or neonatal T cells after activation will be necessary to establish if this hypothesis is correct. In addition, the finding that Thy-1 and the TCR share a physical association with the tyrosine phosphatase CD45 suggest a novel pathway of transmembrane signalling in T cells. Such a model would explain previously puzzling observations on the requirement for co-expression of the TCR on the cell surface, and suggest means of modifying cellular activation by manipulation of CD45.

PUBLICATIONS

Ashwell JD, Jenkins MK, Schwartz RH. Effect of gamma radiation on resting B lymphocytes. II. Functional characterization of the antigen-presentation defect. J Immunol 1988;141:2536-44.

Mercep M, Noguchi PD, Ashwell JD. The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different Ca^{2+} requirements and sensitivity to cyclosporine A. J Immunol 1989;142:4085-92.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09309-03 OAD

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Diversity of γδ T Cells from Different Sources

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	A. M. Kruisbeek	Visiting Scientist	OAD, NCI
Others:	L. Jones	Fellow	OAD, NCI
	M. A. Weston	Biologist	OAD, NCI

COOPERATING UNITS *(if any)*
Laboratory of Immunology, NIAID (R. N. Germain); Massachusetts Institute of Technology (D. H. Raulet); Whitehead Institute (A. Korman); Netherlands Cancer Institute (J. Borst)

LAB/BRANCH
Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.8	1.5	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Most antigen-specific murine and human T cells express a polymorphic receptor, consisting of two disulfide linked chains, termed α and β, noncovalently linked to a nonpolymorphic complex of proteins, termed T3. A second type of T cell, expressing a T3 complex linked to two new chains, termed γ and δ, was discovered a few years ago. These cells most often express a double-negative (CD4-CD8-) phenotype and are predominantly expressed in early T cell populations. To achieve a better understanding of the expression of this new receptor at the clonal level, panels of T-cell hybridomas with γδ receptors were established from a variety of sources, i.e., adult thymus, fetal thymus, nude spleen, and nude spleen after thymus transplantation. Biochemical analysis of T-cell hybridomas from adult thymus has demonstrated so far usage of three different species of γ chain and four different species of δ chain, thus revealing a diversity in both γ and δ not expressed at the total population level. Comparisons of variable region gene usage for both the δ and γ chain for γδ receptors on cells derived from different sources is currently in progress, and various novel types of rearrangements have already been identified. The study is aimed at acquiring a complete picture of the diversity of the expressed γδ repertoire, which will involve a combination of biochemical and molecular analysis of the established cell lines. The significance of this project lies in the understanding in the role of γδ cells in early T-cell development.

PHS 6040 (Rev 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD, NCI
Lori Jones	Fellow	OAD, NCI
Margaret A. Weston	Biologist	OAD, NCI

OBJECTIVES

1. To study expression of the T_3-γ-δ-TCR-complex at the clonal level, and to compare the spectrum of functional activities expressed by these cells with those of conventional $\alpha\beta$ cells;

2. To develop clones and hybridomas of antigen-specific T_3-γ-δ cells from different lymphoid sources and compare variable region gene usage;

3. To develop monoclonal antibodies against γ and δ proteins, which will be used to explore the effect of in vivo and in vitro elimination of cells expressing the $\gamma\delta$ receptor; and

4. To understand the physiologic role of T_3-γ-δ cells in the developing thymus and in the peripheral lymphoid tissues.

MAJOR FINDINGS

Since the identification of the protein products of the γ gene family on a new type of T cells in both mouse and man, much has been learned about their expression. The γ chain is, on most γ-expressing T cells, associated with a partner chain, termed δ. The $\gamma\delta$ heterodimer is associated with an invariant T_3-complex, appears predominantly on cells with CD4-CD8- phenotype both in the thymus and the periphery, and the activation properties of cells expressing this receptor appear similar to those of conventional $\alpha\beta$ T cells. Knowledge of the potential diversity of T_3-$\gamma\delta$ cells is essential to our understanding of the physiologic role of this distinct T-cell lineage. We are currently comparing the V-region gene usage in $\gamma\delta$-T cell clones and hybridomas established from different sources (adult and fetal thymus, peripheral lymphoid organs), and establishing the role of the thymus in generating diversity in the $\gamma\delta$-T cell repertoire (through a comparison of V-region gene usage in T cells from nude mice with and without thymus grafts). Furthermore, receptor material isolated from these cell lines is used to raise monoclonal antibodies. These antibodies will be used to explore the effect of in vivo and in vitro elimination of cells expressing the $\gamma\delta$ complex on normal T-cell development. Finally, the responsiveness of $\gamma\delta$ cells against various mycobacterium antigens is analyzed in relationship to the type of γ and δ chain expressed, in view of recent reports on responses to mycobacteria by particular $\gamma\delta$ T cells (see: Holoshitz J, et al.: Nature 1989;339:226-9 and Augustin A, et al.: Nature 1989;340:239-41).

PUBLICATIONS

Korman AJ, Marusic S, Spencer D, Kruisbeek AM, Raulet DH. Limited repertoire of variable genes expressed by $\gamma\delta$ T cell receptor bearing cells in the adult thymus. J Exp Med 1988;168:1021-44.

Marusic S, Saito T, Tentori L, Zuniga J, Allison JP, Raulet DH, Kruisbeek AM. A novel $\gamma\delta$ TCR for antigen adds limited diversity to the $\gamma\delta$ repertoire in the adult thymus. J Immunol 1989;142:28-34.

Saito T, Hochstenbach F, Marusic S, Kruisbeek AM, Brenner M, Germain RN. Surface expression of only $\gamma\delta$ and/or $\alpha\beta$ T cell receptor heterodimers by cells with four (α,β,γ,δ) functional receptor pairs. J Exp Med 1988;168:1003-20.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09310-03 OAD

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Early T Cell Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. M. Kruisbeek	Visiting Scientist	OAD, NCI
Others:	M. A. Weston	Biologist	OAD, NCI
	J. Zuniga-Pflucker	General Fellowship Program	OAD, NCI
	J. Fine	Fellow	OAD, NCI

COOPERATING UNITS *(if any)*
EIB, NCI (D. Singer); LEI, BRMP, NCI-FCRF (H. Young)

LAB/BRANCH
Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.6	0.8	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The signals and growth factors required for the development of T cells are largely unknown. During early fetal thymus ontogeny, a variety of cell surface molecules are expressed which could potentially serve as transducers of activation signals. Two such molecules (i.e., Thy-1 and the epsilon-component of the T3 complex) were explored and shown to function in early fetal thymocyte activation, as evidenced by both induction of proliferation and elaboration of lymphokines. We found that both interleukin-2 (IL-2) and BSF-1/IL-4 are produced upon activation of fetal thymocytes through Thy-1 and T3-activation, and are currently exploring, in an in vitro organ culture system, the effect of blocking IL-2 or IL-4 usage on T-cell development, as well as designing transgenic mice with a disability in IL-2 production. These, as well as other lymphokines currently investigated, could play a role not only as growth factors, but, as preliminary studies indicated, also as differentiation factors. The potential role of the IL-2 and IL-2 receptors (IL-2R) pathway is not limited to the fetal thymus: also the regenerating thymus after radiation and after bone marrow transplantation goes through a stage in which the majority of the CD4-CD8- cells express IL-2R. Finally, the functionality of IL-2R was determined by Scatchard-plot analysis; the affinities of IL-2R on developing thymocytes (a high-affinity and a low-affinity) were remarkably similar to those on IL-2-dependent cell lines.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD, NCI
Margaret A. Weston	Biologist	OAD, NCI
Juan Zuniga-Pflucker	General Fellowship Program	OAD, NCI
Jay Fine	Fellow	OAD, NCI

OBJECTIVES

1. To determine what signals determine intrathymic differentiation of T cells during fetal ontogeny, i.e., which receptor-ligand interactions and lymphokines are utilized as signals for expansion and differentiation of fetal thymocytes; and

2. To explore in vivo and in vitro models in the effects of blocking IL-2 and IL-4 usage on T-cell development.

MAJOR FINDINGS

The cellular and humoral factors required for intrathymic growth and differentiation of fetal T cells are largely unknown. We recently found that early fetal thymocytes can utilize both the Thy-1 molecule and the T_3-complex as transducers of activation signals. In addition, we demonstrated that fetal thymocytes produce both IL-2 and IL-4 upon activation. As these studies were performed at a timepoint before conventional $\alpha\beta$ receptor-bearing cells are present, the results suggest a role for early $\gamma\delta$ receptor bearing cells in development, i.e., production of lymphokines. Current studies focus on: (1) which other cell surface molecules are involved in the delivery of successful activation signals to early T cells; and (2) which role the $\gamma\delta$ cells play in the development of $\alpha\beta$ cells.

PUBLICATIONS

Tentori L, Longo DL, Zuniga-Pflucker JC, Wing C, Kruisbeek AM. Essential role of the interleukin 2-interleukin 2 receptor pathway in thymocyte maturation in vivo. J Exp Med 1988;168:1741-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09311-03 OAD

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antigen-Specific Receptor Structure and Function in T Lymphocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. D. Ashwell	Senior Investigator	OAD, NCI
Others:	M. Mercep	Visiting Fellow	OAD, NCI

COOPERATING UNITS *(if any)*
Laboratory of Cell Biology and Metabolism, NICHHD (A. Weissman, J. O'Shea, and R. Klausner)

LAB/BRANCH
Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.5	2.5	.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The TCR comprises seven chains and exists in two forms on the majority of peripheral cells. Loss of the ζ chains results in a marked decrease in levels of the other TCR components on the cell surface. Transfection of these cells with ζ chain cDNA resulted in reconstitution of both total cellular ζ levels as well as cell surface TCR levels. Modulation of transfected ζ chain expression with sodium butyrate resulted in similar modulation of cell surface TCR, proving that the ζ chain is the limiting TCR component in these cells. Although transfection wit ζ cDNA restored ζ chain expression, the η chain could not be detected in these cells. The transfectants expressed on the ζ2-containing form of the TCR, not the ζη-containing form. Analysis of both early and late biological consequences of activation with antigen revealed the following: in contrast to their ζη-normal counterparts, ζη-cells manifested little or no phosphoinositide hydrolysis after activation. In spite of this, these cells produced normal levels of IL-2 and were blocked in the cell cycle between the G1 and S phases. When activation-induced cell death was measured, it was found that ζη- cells did not lyse. These results suggest the ζη and ζ2-bearing TCRs have distinct biological functions. The failure of ζη- cells to lyse after activation may implicate this form of the TCR in thymic negative selection. In another series of experiments, a T-cell hybridoma was transfected with the v-src oncogene, a tyrosine kinase. Successful transfectants were found to have spontaneous phosphorylation patterns similar to those seen in the activated wild type cell. These transfectants, but not mock transfectants, also produced IL-2 spontaneously. As with v-src expression itself, sodium butyrate was able to upregulate IL-2 production. These results prove that tyrosine kinase activation, in the apparent absence of PI hydrolysis or protein kinase C activation, is able to lead to IL-2 production. In conjunction with our finding that this pathway is intact in the ζη- transfectants, these results suggest that tyrosine kinase activation is a major physiological pathway in T cell leading to lymphokine secretion.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Investigator	OAD, NCI
Mladen Mercep	Visiting Fellow	OAD, NCI

OBJECTIVES

(1) To analyze the structural and functional roles of the different components of the T cell receptor antigen-specific receptor;

(2) To determine which second signals, such as phosphatidylinositol metabolism or increases in intracellular free Ca^{2+}, are involved in T cell activation; and

(3) To distinguish, if possible, those signals that lead to lymphokine production and those that lead to the death of transformed T cells.

MAJOR FINDINGS

I. TCR ζ_2 and $\zeta\eta$ as Subunits Involved in Transmembrane Signalling

The T cell antigen-specific (TCR) has three genetically related subunits: the antigen-binding heterodimer (Ti) composed of the α and β chains, the CD3 complex composed of the γ, δ, and ϵ chains, and the ζ (and probably η) chains. Each mature T cell has two structurally different TCR complexes, one (~80% of TCR's) that has a ζ chain homodimer (ζ_2), and another that has a ζ chain disulfide-linked to an η chain ($\zeta\eta$). We have previously described T cell TCR variants that have lost either the ζ chain. These cells expressed very low levels of the other TCR chains on the cell surface, due to rapid and efficient degradation in lysosomes. To prove that it was the loss of ζ that resulted in this phenotype, in collaboration with Drs. Allan Weissman and Richard Klausner, ζ chain cDNA has been transfected back into these cells. Successful transfectants expressed levels of ζ similar to the wild type, and importantly, expressed normal levels of most of the other chains of the TCR on the cell surface. In fact, upregulating transcription of the ζ chain with sodium butyrate resulted in a direct increase in cell surface TCR expression. These results demonstrate that the ζ chain is limiting in the assembly of the TCR in T cells with a mature phenotype, and that in its absence most of the TCR components are rapidly degraded. An unexpected and intriguing finding in these cells was that transfection with ζ failed to restore any detectable η expression. Therefore, these cells provide for the first time a source of T cells bearing exclusively the ζ_2 form of the TCR. As we had found previously with $\zeta\eta$ "low" T cell mutants, phosphatidylinositol (PI) hydrolysis in response to antigen stimulation was virtually absent in these cells. Cross-linking antibodies to the TCR also generally gave a reduced response in this assay, although one $\zeta\eta^-$ clone was almost identical to the wild type cell. Extensive dose-response curve analysis showed that this was a qualitative, not a quantitative, difference, since this same cell failed to generate PI hydrolysis even at extremely high levels of antigen stimulation. We had previously been unable to determine the functional consequences of $\zeta\eta$ loss, since the mutant cells failed to secrete interleukin-2 (IL-2) even in response to pharmacologic stimuli that bypass the TCR. The $\zeta\eta$- transfectants, however,

produced substantial amounts of IL-2 in response to PMA and ionomycin. Antigen stimulation of these cells as well as other, $\zeta\eta$-*normal*, subclones revealed that all made IL-2 with similar dose-response curves. Activation-induced growth inhibition, the manifestation of a G_1/S cell cycle block, was also initiated normally in these cells in the absence of $\zeta\eta$. However, the typical lytic response to antigen stimulation was markedly reduced in the $\zeta\eta^-$ cells, although it was present in all $\zeta\eta$-normal cells tested. These data prove that the TCRs on these cells recognize antigen. Moreover, they strongly support a model that holds the TCR has two discrete forms that couple to signal transduction pathways. The ζ_2 form couples predominantly to a tyrosine kinase pathway, and its occupancy results in both IL-2 production and G_1/S cell cycle block. The $\zeta\eta$ form couples to a PI/Ca^{2+}/protein kinase C pathway. Its occupancy results in cell death (whether it also results in IL-2 production a G_1/S cell cycle block is unknown). It is tempting to speculate that it is this pathway that is involved in negative selection in the thymus. Future work will involve the analysis of transfectants expressing site-directed ζ chain mutations to determine which portions of this structure are responsible for signal coupling.

II. Tyrosine Kinases and Signal Transduction

Much attention has been focused on PI hydrolysis and Ca^{2+} fluxes as critical second messengers in signal transduction leading to IL-2 secretion. Based upon our work with TCR mutants, we have found no correlation between these events. There is another signalling pathway, however, whose role in IL-2 secretion has not been tested. Therefore, we studied the biological consequences of spontaneous tyrosine kinase activity on T cell responses. Drs. John O'Shea and Richard Klausner transfected the 2B4.11 T cell hybridoma with *v-src*, a membrane-associated tyrosine kinase. The pattern of spontaneous phosphorylation of intracellular substrates in these cells was identical to that obtained in normal 2B4.11 cells after stimulation with antigen or mitogenic antibodies. Analysis of the supernatants from many *v-src* transfectants found that they contained T cell growth factors even in the absence of stimulation. Assays in the presence of the appropriate antibodies allowed us to identify the biologically active material as IL-2. Importantly, increasing the level of *v-src* activity with sodium butyrate, which enhances the transcription of many transfected genes, increased the amount of IL-2 secreted in parallel. In fact, the levels of IL-2 that were achieved with sodium butyrate approached those induced by a mitogenic anti-Thy-1 monoclonal antibody. No IL-2 was observed in untransfected cells or in cells that received the retroviral construct without *v-src* under any conditions. These results directly implicate tyrosine kinase activity in the IL-2 induction pathway, and indirectly confirm the importance of the ζ_2-containing TCR in this event.

SIGNIFICANCE

These studies broaden our understanding of the relationship between TCR structure and function. They strongly suggest that rather than existing as a monolithic structure with all-or-none functional properties, the TCR consists of multiple functional subunits or domains, that couple independently to different intracellular events. This insight may help to explain the paradox of how activation causes T cell deletion in the thymus but T cell expansion in the periphery. Further, the previously assumed role of PI hydrolysis and Ca^{2+}

fluxes, shown here to be largely dependent upon one form of the TCR, cannot explain the ability of the T cells in these studies to product biologically important lymphokines. Rather, they support the hypothesis that the relatively recently described phenomenon of tyrosine kinase activation may be the critical second messenger. Such analyses are required to ultimately understand how this complex receptor determines the myriad biological phenomena associated with T cell activation.

PUBLICATIONS

Bonifacino JS, Chen C, Lippincott-Schwartz J, Ashwell JD, Klausner RD. Subunit interactions within the T cell antigen receptor: clues from the study of partial complexes. Proc Natl Acad Sci USA 1988;85:6929-33.

Bonifacino JS, Mercep M, Sussman JJ, Klausner RD, Ashwell JD. The T-cell antigen receptor: a complex signal-transducing molecule. In: Hamaoka T, et al., eds. Immune System and Cancer. Tokyo: Japan Sci Soc Press, 1989:87.

Mercep M, Bonifacino JS, Garcia-Morales P, Samelson LE, Klausner RD, Ashwell JD. T cell CD3-$\zeta\eta$ heterodimer expression and coupling to phosphoinositide hydrolysis. Science 1988;242:571-4.

Sussman JJ, Mercep M, Saito T, Germain RN, Bonvini E, Ashwell JD. Dissociation of phosphoinositide hydrolysis and Ca^{2+} fluxes from the biological responses of a T cell hybridoma. Nature 1988;334:625-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09323-02 OAD

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Selection of the T Cell Repertoire

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. M. Kruisbeek	Visiting Scientist	OAD, NCI
Others:	M. A. Weston	Biologist	OAD, NCI
	L. Jones	Fellow	OAD, NCI
	J. Zuniga-Pflucker	General Fellowship Program	OAD, NCI

COOPERATING UNITS *(if any)*
Experimental Immunology Branch, NCI (A. Singer)

LAB/BRANCH
Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During differentiation in the thymus, T cells develop the receptor repertoire which allows them to recognize antigen in the context of self major histocompatibility complex (MHC) molecules. Thymic MHC-encoded determinants greatly influence the selection of the T-cell receptor repertoire. Both positive and negative selection is thought to occur in the thymus, but how this "education" occurs is not well understood. It has been suggested that an interaction between the T-cell receptor (TCR) and MHC-encoded determinants occurs, leading to the selection of an MHC-restricted receptor repertoire. Our work has confirmed this hypothesis, and is currently focussing on the additional role that CD4- MHC and CD8- MHC interactions exert on the selection process. Repertoire analysis is performed by testing for expression of particular TCR-chains (Vβ3-5-6-8-11-17) that can be identified with monoclonal antibodies. Expression of these chains is associated with recognition of particular MHC antigens, while deletion of T cells with these TCR's occurs when other defined MHC antigens are expressed. Extrathymic T-cell repertoire development is also being analyzed, using the models of neonatally thymectomized mice and nude mice. Such an analysis will not only yield a formal answer to the question of how the thymus affects tolerance induction, but also yield insights into the thymus-independent T-cell repertoire. This pathway of development appears to be a prominent one, especially in adult patients after bone marrow transplantation. The significant of this project lies in: (1) understanding the factors that control development of T cells; and (2) applying this knowledge to restoring T cells after bone marrow transplantation and controlling tolerance induction.

PHS 6040 (Rev 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD, NCI
Margaret A. Weston	Biologist	OAD, NCI
Lori Jones	Fellow	OAD, NCI
Juan Zuniga-Pflucker	General Fellowship Program	OAD, NCI

OBJECTIVES

To determine the role of CD4, CD8, TCR and MHC antigens during T-cell development.

MAJOR FINDINGS

Blocking of expression of MHC antigens leads to a failure to develop mature T cells. Subsequent studies focus on the cause of this phenomenon, i.e., is it due to blocking a positive signal delivered through TCR-MHC interactions or through accessory molecule-MHC interactions or both. Studies aimed at selectively blocking some, but not other, MHC determinants demonstrated a firm role for TCR-MHC interactions in the selection of the T cell repertoire. However, these clearly are not the only type of required interactions: indeed, both CD4 and CD8 molecules were shown to be involved in positive selection as well.

PUBLICATIONS

Kruisbeek AM, Zuniga-Pflucker J, Marusic S, Weston MA, Tentori L, Longo DL. Thymic selection of the T cell repertoire. Immunol Res 1988;7:318-28.

Marusic S, Longo DL, Kruisbeek AM. Preferential differentiation of T cell receptor specificities based on the MHC glycoproteins encountered during development. J Exp Med 1989;169:1619-30.

McCarthy SA, Kruisbeek AM, Uppenkamp IK, Sharrow SO, Singer A. Engagement of the CD4 molecule influences cell surface expression of TCR by developing thymocytes. Nature 1988;336:76-9.

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, Singer A. Differentiation of Ia-reactive $CD8^+$ murine T cells does not require Ia engagement. J Exp Med 1988;168:437-42.

Zuniga-Pflucker J, Longo DL, Kruisbeek AM. Positive selection of $CD4^-CD8^+$ T cells in the thymus. Nature 1989;338:76-8.

Zuniga-Pflucker J, McCarthy SA, Weston M, Longo DL, Singer A, Kruisbeek AM. Role of CD4 in thymocyte selection and maturation. J Exp Med 1989;169:2085-96.

SUMMARY REPORT

BIOLOGICAL RESOURCES BRANCH

October 1, 1988 Through September 30, 1989

INTRODUCTION

The Biological Resources Branch (BRB) is the extramural arm of the Biological Response Modifiers Program (BRMP).

The BRB supports preclinical and clinical biological response modifiers (BRMs) research in the biomedical community through a program of grants and contracts. The Branch sponsors Phase I and early Phase II clinical studies. These studies assess biological effects of BRMs and correlate changes in the biological responses with antitumor activity. A repository distributes selected agents for preclinical studies performed by National Institutes of Health (NIH) supported intramural and extramural investigators. Other contracts support the production and in vivo evaluation of monoclonal antibodies, immunoconjugates and other BRMs.

OFFICE OF THE CHIEF

Chief, BRB	Stephen P. Creekmore, Ph.D., M.D.
Program Analyst	Alfred C. Denn
Program Analyst	Paul J. Miller
Secretary	Virginia L. Axline
Clerk-Typist	Dawn M. Crigger

PRECLINICAL STUDIES GROUP

Ira Green, M.D.
Toby T. Hecht, Ph.D.
Morris I. Kelsey, Ph.D.
Craig W. Reynolds, Ph.D.

CLINICAL TRIALS GROUP

Stephen P. Creekmore, Ph.D., M.D.

BIOLOGICAL RESOURCES BRANCH, PRECLINICAL STUDIES GROUP

This group is responsible for monitoring the results of BRMP-supported preclinical research as well as testing, production and development contracts supported by the BRMP. Project Officers on all BRB contracts in the preclinical area are a member of this group, as are the Program Directors for all grants in the BRB. This group also identifies and acquires BRMs of interest to the BRMP through its relationships with extramural laboratories and biotechnology companies. Development of potential agents is coordinated with appropriate program personnel and working groups, such as the BRMP Operating Committee (OC), BRMP-CTEP Working Group (BCWG), and the BRMP-DTP Working Group (BDWG).

BIOLOGICAL RESOURCES BRANCH, CLINICAL TRIALS GROUP

The BRB Clinical Trials Group initiates and monitors Phase Ia and Ib clinical trials of BRMs through contracts, e.g., Master Agreement Orders. An important object of these trials is the correlation between changes in immunological reactivity and clinical efficacy and toxicity in these studies. This group maintains close liaison with the Investigational Drug Branch and the Regulatory Affairs Branch of the Cancer Therapy Evaluation Program (CTEP) of the Division of Cancer Treatment, to coordinate appropriate development of investigational agents for clinical trials, and for regulatory issues.

Coordination of the clinical development of BRMs through preclinical studies and pilot clinical trials is done jointly with CTEP personnel through the BRMP-CTEP Working Group (BCWG). This Group also coordinates efforts with CTEP in the planning and review of Phase III clinical trials which include BRMs.

SUMMARY OF FY 89 ACTIVITIES

During this year the BRB has been involved in the following:

* Maintained 26 contracts for testing BRMs in Phase I clinical trials.

* Awarded two new 5-year contracts for Phase I clinical trials of BRMs, "Task A monoclonal antibodies, and other targeting molecules."

* Awarded one additional contract and maintained three existing 5-year contracts for Phase I clinical trials of BRMs, "Task B-cytokines and other immune modulators."

* Produced or procured over 300 grams of preclinical and clinical grade monoclonal antibodies in support of NIH supported investigations.

* Established the capability for chelation of monoclonal antibodies for clinical studies of imaging and radiotherapy.

* Expanded the distribution of biological standards to include a battery of international standards in collaboration with the National Biological Standards Board Repository in England. The standards now supported include interleukin (IL)-1 alpha, IL-1 beta, IL-4, IL-6, tumor necrosis factor (TNF)-alpha, granulocyte/macrophage-colony stimulating factor (GM-CSF), as well as the IL-2 standard already being distributed.

* During FY 89, the BRB awarded $5.2 million for extramural contracts, including $3.2 million in clinical contracts and $2.0 million in preclinical contracts.

GRANTS AND CONTRACT ADMINISTRATION

The BRB provides planning, direction, implementation, and evaluation of research supported by grants and contracts. The Branch staff maintains liaison with all pertinent peer review groups involved in grant and contract review.

Liaisons have been established and maintained with other programs in the NCI, including the Immunology and Tumor Biology Programs in the Division of Cancer Biology and Diagnosis (DCBD), to minimize overlap in the grant and contract areas. A regular working relationship is maintained with the Developmental Therapeutics Program (DTP) of the Division of Cancer Treatment (DCT) to coordinate drug and biologic development. A cooperative clinical protocol evaluation system with the CTEP, DCT has been established to coordinate Phase I, Phase II, and Phase III BRM clinical trials. This includes membership of several staff members of the CTEP on the BRM Decision Network Committee (DNC) and the establishment of a BCWG.

ACCOMPLISHMENTS OF THE BRMP GRANT PROGRAM IN FY 89

A total of 97 grants were supported by the BRMP with $26.7 million during the period October 1, 1988 through August 15, 1989. There were 66 Research Projects (R01), 11 Program Project (P01), 1 Outstanding Investigator Grant (R35), 2 Phase I SBIR (R43), 2 Phase II SBIR (R44), 8 Merit Awards (R37), 3 Conference Grants (R13), 4 First Investigator Research Grant (R29)
(Tables I & II).

Approximately one-third of current BRMP-monitored grants address the topic of the development and/or use of monoclonal antibodies in cancer treatment. For example, 7 out of 10 Program Project grants are in the area of monoclonal antibodies. The major disease systems being studied, both preclinically and clinically, are: melanoma, leukemia/lymphoma, colon, renal cell and breast cancer. These studies employ unconjugated antibodies as well as toxin or radionuclide-conjugated antibodies with or without the addition of lymphokines.

In the area of melanoma research, one Program Project has been funded to study the clinical use of monoclonal antibodies to disialoganglioside antigens (GD2 and GD3) present on melanoma cells. These antibodies have been shown to cause an inflammatory reaction at the site of the tumor and may play an important role in observed tumor regressions. These antibodies will now be combined with cytokines and the mechanism of antitumor response will be investigated. One BRMP-sponsored Outstanding Investigator Award supports the development of new useful monoclonal antibodies for melanoma and the testing of these antibodies, chimeric constructs, and chemoimmunoconjugates in relevant animal models including the reconstituted severe combined immunodeficient (SCID) mouse. Another investigator is studying the generation of anti-idiotype antibodies to an anti-melanoma associated antigen (p97). Some of these anti-idiotype antibodies act as "internal images" of the p97 antigen and may have antitumor activity in a mouse model. In addition, human monoclonal antibodies to melanoma antigens are being prepared by an investigator who is using these reagents for intralesional inoculation in melanoma patients. In the area of active specific immunotherapy, melanoma vaccines are being

prepared and tested in the clinic by one grant-supported scientist, while another investigator is using purified antigens with several different adjuvants for immunization.

Leukemia/lymphoma research has focused in part on the use of conjugated monoclonal antibodies and anti-idiotypic monoclonal antibodies in the clinic and in relevant animal model systems. In one Program Project grant, investigators are using the I131-conjugated monoclonal antibody Lym-1 with a great deal of success to treat patients with B-cell lymphoma. In the same grant, new metal chelators are being developed in order to use other radioisotopes such as Cu67 for conjugation. In another Program Project grant which deals with the use of radioiodinated monoclonal antibodies for the treatment of leukemia, idiotypes and differentiation antigens are used as antibody targets. These studies include the possibility of bone marrow transplantation when necessary. Other investigators are using unconjugated anti-idiotypic antibodies for B-cell and T-cell lymphomas. Immunotoxin development continues to be an important focus of leukemia/lymphoma research in the BRMP grant program.

Drug and BRM delivery is an area of great relevance for study. Several grants are concerned with the use of liposomes to deliver BRMs and drugs. In some cases, the liposomes were conjugated with monoclonal antibodies so that specific targeting of tumor cell was possible. In other cases, liposomes were used to deliver an immune modifier to the reticuloendothelial system so that activation of monocytes resulted. Research in this area ranges from the very basic questions of chemical composition, charge and size of the liposome for adequate pharmacokinetics and biodistribution to the clinical testing of liposomes containing BRMs to eliminate lung metastases in childhood osteogenic sarcoma.

In the area of adoptive immunotherapy, one important problem is the ex vivo growth of sufficient quantities of specific cells for treatment. Several grants deal with this problem using antibodies or phorbol esters to stimulate in vitro growth of tumor-specific cells. In other research, the problem of developing specific immunity to poorly immunogenic and non-immunogenic tumors is being approached.

In the area of cytokine research, determination of the mechanism of action of cytokines is proceeding with the goal of designing therapeutic approaches which may also include antibodies and/or cytotoxic drugs. There are also preclinical studies on the nature of B-cell activating and differentiating factors which may ultimately benefit clinical investigations. Mullerian Inhibiting Substance is being tested as an antitumor agent for malignancies arising from the Mullerian duct.

Molecular biological techniques are being used as important tools for the construction of novel targeting agents for cancer therapy. Small linear molecules which contain the Fv region of antitumor monoclonal antibodies are being produced efficiently in bacterial systems and are being tested in vitro and in mouse model systems. In addition, hormone/toxin hybrid molecules are being constructed which target cells that have specific receptors. One particular hybrid molecule is now being studied clinically in patients with T-cell lymphomas. Another novel approach to cancer therapy involves the use of

tissue-specific promoters and enhancers to regulate selectively the expression of inserted genes coding for toxin molecules.

On November 3-4, 1988, a 2-day workshop was held in Frederick, Maryland on the "Establishment of Standards for Recombinant Human Cytokines." This workshop was co-sponsored by the BRMP, the Food and Drug Administration (FDA) and the National Institutes of Allergy and Infectious Diseases (NIAID). One hundred and twenty-five participants discussed a variety of practical issues in the development and distribution of recombinant cytokine standards for basic research and industrial product development.

A 3-day conference, jointly sponsored by the BRMP and NIAID, entitled "Oligonucleotides as Antisense Inhibitors of Gene Expression: Therapeutic Applications," was held on June 18-21, 1989 in Rockville, Maryland. Several hundred people participated in this conference that provided the theoretical basis for the eventual use of these materials in patients.

A 2-day workshop on "Regulatory Issues for Hematopoietic Growth Factors (HGF)" was held on May 8-9, 1989 in Bethesda, Maryland. This meeting was co-sponsored by the BRMP, FDA, NIAID and the National Heart, Lung and Blood Institute (NHLBI), and was attended by over 125 individuals from nearly 50 companies. The purpose of this meeting was to discuss a variety of issues related to the rapid and efficient movement of HGFs from preclinical testing into clinical trials.

Request for Applications

In FY 89, the BRMP issued one new RFA: "Studies of Chronobiological Effects in Cancer Treatment With Biological Response Modifiers and/or Drugs." A total of 14 applications have been received. The review of these applications will take place July 1989. It is expected that two grants will be funded in FY 90. Of the three RFAs issued in FY 87, "Manipulation of the Suppressor Arm of the Immune Response Directed Towards Successful Human Immunotherapy," "Protection of Bone Marrow Against the Effects of Cytotoxic Drugs and X-Irradiation," and "Immunologic Investigation of Multidrug Resistance," 4 out of 47 applications have been funded and one additional application will be funded this year as a result of a review of an amended grant application.

Program Announcements

In FY 86 the BRMP issued six Program Announcements: Development of New Methods to Couple Cytotoxic Agents to Monoclonal Antibodies; Preclinical Studies of LAK Phenomenon; Determination of the Therapeutic Usefulness of Purified Cytokines in Cancer Models; Use of Oncogene Related Products for Cancer Therapy; Application of Neuroendocrine Effects on the Immune System for Cancer Therapy; and Determination of the Therapeutic Usefulness of Maturation, Differentiation and Anti-Growth Factor Substances in Cancer Models. Grant applications are still being received for these Program Announcements and six others issued in the past.

In FY 89, the BRMP and DTP issued one Program Announcement: Specific Cancer Cell Targeting Using Molecular Genetic Technology. Grant applications are currently being received in response to this Program Announcement and to the six previously issued Program Announcements. In FY 89, 10 grant applications were received and one of these applications has been funded related to the topic Determination of

the Therapeutic Usefulness of Maturation, Differentiation and Anti-Growth Factor Substances in Cancer Models.

BRMP CONTRACT PROGRAM

The BRB put out a Request for Proposals (RFP) for oligonucleotides production and received five responses. The source selection is expected to be with two or more of these Master Agreement holders. An RFP for chimeric monoclonal antibodies was also released with source selection being held in the third quarter of FY 89 for production of chimeric antibody 225 and R24.

Two Task A contracts for Phase I clinical studies of monoclonal antibodies and immunoconjugates were placed with the University of Alabama, and the University of Texas, M.D. Anderson Cancer Center, and one Task B contract with Cleveland Clinic for Phase I clinical studies of cytokines (Table III).

The BRB maintained 26 clinical Task Order contracts with 15 extramural institutions for testing BRMs in Phase I clinical trials (Table IV).

The BRB continued the distribution of IL-2 reference standard to the scientific community through the expanded function of the BRMP preclinical repository at Hazleton Laboratories in Vienna, Virginia. The BRB continued to solicit for BRMs from private industry for distribution to intramural and extramural preclinical investigators in order to stimulate innovative preclinical research in BRMs. The number of requests processed for biologicals increased at a rate of 25-30% per month. Additionally, the repository was expanded to include international standards from the National Biological Standards Board Repository in England.

During this fiscal year, the Branch recommended Small Business Innovative Research (SBIR) awards, resulting in the funding of two Phase I and one Phase II SBIR contract (Table V).

BRB staff reviewed and visited all required preclinical contracts during the fiscal year.

The BRB continued its collaboration with the CTEP in the BCWG, which serves an expanding role coordinating the biological response modifier drug development programs of BRMP and CTEP. Representatives of National Institute of Allergy and Infectious Diseases (NIAID) also participated when Acquired Immune Deficiency Syndrome (AIDS)-related issues were discussed.

The Program has also continued work with the DTP in the BRMP/DTP Working Group (BCWG), which met to discuss the BRMP and the DTP functions in development of new BRMs for cancer and AIDS therapy.

PHARMACEUTICAL COMPANY RELATIONSHIPS

The BRMP has established relationships with most of the biotechnology and pharmaceutical companies which produce BRMs. Relationships exist with Burroughs-Wellcome, Hoffmann-LaRoche, Schering, Biogen, Cetus-Shell, Genentech, Collaborative Research, Hybritech, Immunex, Becton Dickenson, Hoechst-Roussel, Dainippon, Sandoz Research Institute, Ortho, Brunswick, Celltech, Lederle (American Cyanamid), Eastman, etc. Each of these companies is currently supplying agents for clinical trials. A number of other companies have supplied

compounds for preclinical evaluation. In some cases, clinical trials evaluating these new compounds are currently being planned or underway.

The BRB is currently expanding its relationships with the biotechnology industry by offering the opportunity to collaborate on joint Government/industry programs for those BRMs with the greatest potential for clinical utility.

NEW INITIATIVES

In addition to the new initiatives already noted in the preceding text, the following areas are being developed:

* RFP announcements, competitions and source selection for funded master agreements to produce reverse sense oligonucleotides, the first of which were held in FY 89.

* RFP announcements, competitions and source selection for funded task orders for the production of chimeric human-mouse monoclonal antibody clones, the first of which were held in FY 89.

* Establishment of 5-year Phase I clinical contracts will provide a stable support for clinical investigations of monoclonal antibodies and cytokines.

* Establishment of a new Master Agreement mechanism for clinical trials involving complex or sophisticated immunological manipulations for the treatment of cancer or AIDS.

BIOLOGICAL RESPONSE MODIFIERS PROGRAM OPERATING COMMITTEE (BRMPOC)

The BRMPOC acts as the steering committee for the BRMP extramural program. The BRMPOC reviews and prioritizes agents as to evaluation in the preclinical screens. The recommendation of the BRMPOC are subsequently presented to the DNC and in turn the recommendations of the DNC are implemented by the BRMPOC and program staff. Members of the BRMPOC are asked, on a rotating basis, to review and summarize data from pharmaceutical firms and present their recommendations on any candidate agent to the Committee.

PUBLICATIONS

Creekmore SP, Harris JE, Ellis TM, Braun DP, et al. A phase I clinical trial of recombinant interleukin-2 by periodic 24-hour intravenous infusions. J Clin Oncol 1989;7:276-84.

Dutcher JP, Creekmore S, Weiss GR, Margolin K, Markowitz AB, Roper M, Parkinson D, Ciobanu N, Fisher RI, Boldt DH, et al. A phase II study of interleukin-2 and lymphokine-activated killer cells in Patients with metastatic malignant melanoma. J Clin Oncol 1989;7:477-85.

Ellis TM, Creekmore SP, McMannis JD, Braun DP, et al. Appearance and phenotypic characterization of circulating Leu 19+ cells in cancer patients receiving recombinant interleukin 2. Cancer Res 1988;48:6597-602.

Fukui H, Reynolds CW, Lenz B, Schneider M, and Talmadge JE. Mechanism of immunotherapeutic activity of OK-432 in the treatment of peritoneal carcinomatosis. Cancer Immun & Immunother, 1989;29:1-6.

Gaynor ER, Vitek L, Sticklin L, Creekmore SP, et al. The hemodynamic effects of treatment with interleukin-2 and lymphokine-activated killer cells. Ann Intern Med 1988;109:953-8.

Gronberg A, Ng J, Ferm MT, Reynolds CW, and Ortaldo JR. Analysis of the mechanism of interferon-induced resistance to NK cell-mediated lysis. In: Ades EW and Lopez C, eds. Natural Killer Cells. Basel Karger, 1989;188-193.

Knoshi N, Ward JM, Reynolds CW, Lijinsky W. Thymic T-cell lymphoma with the $CD8^+$ (OX-8, $CD4^+$ (W3/25) phenotype, induced in F344/NCr rats by nitroso-2-hydroxypropylurea. Thymus; in press.

Kozeny GA, Nicolas JD, Creekmore S, Sticklin L, Hano JE, Fisher RI. Effects of interleukin-2 immunotherapy on renal function. J Clin Oncol 1988;6:1170-6.

McMannis JD, Fisher RI, Creekmore SP, Braun DP, Harris JE, Ellis TM. In vivo effects of recombinant IL-2. I. Isolation of Circulating Leu-19+ lymphokine-activated killer effector cells from cancer patients receiving recombinant IL-2. J Immunol 1988;140:1335-40.

Reynolds CW and Hiserodt JC. Summary of Session V: recognition and lysis of targets. In: Ades EW and Lopez C, eds. Natural Killer Cells. Basel Karger, 1989;164-166.

Sayer TJ, Twilley TA, Bull CA, Denn III AC, Pilaro AM, Lokesh B. Effect of cytokines on polymorphonuclear neutophil (PMN) infiltration in the mouse: prostaglandin- and leukotriene-independent induction of infiltration by interleukin 1 and tumor necrosis factor. J Immunol 1989;73:1130-41.

Wiltrout RH, Pilaro AM, Gruys ME, Talmadge JE, Longo DL, Ortaldo JR, Reynolds CW. Augmentation of mouse liver-associated natural killer activity by biologic response modifiers occurs largely via rapid recruitment of large granular lymphocytes from the bone marrow. J Immunol 1989;143.

Wiltrout RH, Reynolds CW, Chirigos MA, Ortaldo JR and Talmadge JE. Induction of NK hyporesponsiveness in blood and spleen by multiple doses of MVE-2 is accompanied by increased NK activity and LGL number in the liver. J Immunol; in press.

TABLE I

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

GRANT EXPENDITURES FOR FY 89 THROUGH AUGUST '89

TYPE OF GRANT	NUMBER	TOTAL COST AWARDED
Research Projects (R01)	66	$10,627,861*
Program Projects (P01)	11	12,382,545
Small Business Innovative Research (SBIR)	4	515,312
Conference Grants (R13)	3	17,500
Merit Awards (R37)	8	2,101,300
First Awards (R29)	4	366,703
Outstanding Investigator Award (R35)	1	687,793
TOTALS	97	$26,699,014*

*Included in this category are 5 Request for Applications (RFA) grants.

TABLE II

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
DEVELOPMENT AND/OR APPLICATION OF MONOCLONAL ANTIBODIES FOR CANCER THERAPY (Grants listed by type of tumor investigated)		
Leukemia and Lymphoma - Preclinical		
CA18105	Applebaum, F. R.	"Immunotherapy Study With Spontaneous Malignancies"
CA39930	Bast, R. C.	"Specific Immunotherapy With Monoclonal Antibodies"
CA26386	Bernstein, I. D.	"Monoclonal Antibody Therapy of Cancer"
CA43735	Herlyn, D.	"Induction of Anti-Tumor Immunity by Anti-Idiotypes"
CA28149	Vitetta, E. S.	"New Strategies for Immunotoxin Therapy"
CA47334	Epstein, A. L.	"Immunotherapy of Human Lymphomas With Lym-1 and Lym-2"
CA47860	Bhattacharya-Chatterjee, M .	"Idiotype Approach to Therapy of Human T-Cell Leukemia"
Leukemia and Lymphoma - Clinical		
CA31888	Ball, E. D.	"Monoclonal Antibodies for the Treatment of Acute Myelogenous Leukemia"
CA44991	Bernstein, I. D.	"Therapy of Leukemia/Lymphoma With Monoclonal Antibodies"
CA47829	Denardo, G. L.	"Studies to Improve Cancer Therapy with Antibodies"
CA33399	Levy, R.	"Human Anti-Tumor Therapy With Monoclonal Antibodies"
CA37497	Royston, I.	"Monoclonal Antibodies in Cancer Detection and Treatment"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
DEVELOPMENT AND/OR APPLICATION OF MONOCLONAL ANTIBODIES FOR CANCER THERAPY		
Leukemia - Clinical (Continued)		
CA41081	Uhr, J. W.	"Immunotoxin Therapy for Patients With B Cell Tumors"
Lung Cancer - Preclinical		
CA33462	Bankert, R. B.	"Monoclonal Antibodies Applied to Treat/Diagnose Cancer"
Ovarian Cancer - Preclinical		
CA37646	Chu, T. M.	"Targeting and Therapy of Tumors With Monoclonal Antibody"
Brain Cancer - Preclinical		
CA29125	Gillespie, G. Y.	"Monoclonal Antibodies to Glioma Cells"
Mammary Tumor - Preclinical		
CA42767	Ceriani, R.	"Monoclonal Antibody Therapy of Breast Cancer"
CA38024	Sirbasku, D. A.	"Monoclonal Antibodies to Mammary Tumor Growth Factors"
Colorectal Cancer - Preclinical		
CA39748	Griffin, T. W.	"Intraperitoneal Immunotoxins"
CA31753	Hawthorne, M. F.	"Boron-10-Labeled Antibodies in Cancer Therapy"
CA37808	Shively, J. E.	"Structure and Genomic Organization of CEA"
CA43904	Shively, J. E.	"Colon Cancer and Engineered Antibodies"
CA33470	Strand, M.	"Chemically Modified Monoclonal Antibody in Tumor Models"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
DEVELOPMENT AND/OR APPLICATION OF MONOCLONAL ANTIBODIES FOR CANCER THERAPY		
Melanoma - Preclinical		
CA38011	Hellstrom, I. E.	"Monoclonal Antibodies to Melanoma Associated Antigens"
Melanoma - Clinical		
CA42508	Reisfeld, R. A.	"Molecular Profile of Melanoma and Neuroblastoma Antigens"
CA30647	Irie, R. F.	"Human Monoclonal Antibody to Ganglioside Antigen"
CA42396	Irie, R. F.	"Treatment of Melanoma With Human Monoclonal Antibody"
CA33049	Oettgen, H. F.	"Monoclonal Antibodies in the Treatment of Cancer"
Anti-Receptor or Anti-Oncogene Product Monoclonal Antibody - Preclinical		
CA42060	Mendelsohn, J.	"Anti-Tumor Activities of Anti-Receptor Antibodies"
CA45187	Roth, J.	"Tumor Antigen Expressed by Oncogene Transformed Cells"
Osteosarcoma-Associated Antigens - Preclinical		
CA43941	Tsang, K. Y.	"Human Monoclonal Antibodies to Human Osteosarcoma Associated Antigens"
Hybrid & Chimeric Antibodies - Preclinical		
CA45232	Khazaeli, M. B.	"Study of Mouse/Human Chimeric Monoclonal Antibodies"
Monoclonal Antibody Localization		
CA43544	Murray, J. L.	"Optimization of Monoclonal Antibody Localization"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
CYCLOPHOSPHAMIDE		
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity By Cyclophosphamide"
Preclinical		
CA30088	Dray, S.	"Synergy of Tumor Chemotherapy and Host Immunity"
MOLECULAR BIOLOGY		
Preclinical		
CA48162	Croop, J. M.	"Immunologic Analysis... Multidrug Resistant Gene Family"
CA45667	Geahlen, R.	"Inhibitors Protein Myristoylation as Anti-Cancer Drugs"
CA42354	Maxwell, I. H.	"Tumor Cell Killing by Expression of a Toxin Gene"
CA41746	Murphy, J. R.	"Diptheria Toxin-Related Peptide Hormone Gene Fusions"
CA40987	Rohrschneider, L. R.	"Tumor Cell Biology of the fms Oncogene Proteins"
AIDS		
CA43447	Bolognesi, D. P.	"Clinical Studies on Prevention and Intervention in AIDS"
LIPOSOMES		
Preclinical		
CA24553	Huang, L.	"Targeting of Liposome to Tumor Cells"
CA37528	Hwang, K. L.	"Controlled Release of Liposomal Contents in Macrophages"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LIPOSOMES - Preclinical (Continued)		
CA42992	Kleinerman, E. S.	"Liposome Therapy--A Potential Adjuvant for Childhood"
CA38043	Lachman, L. B.	"Biological Studies of Human Interleukin-1"
CA39448	Matthay, K. K.	"Treatment of Bone Marrow by Antibody-Directed Liposomes"
CA25526	Papahadjopoulos, P. D.	"Liposome Targeting to Tumor Cells In Vivo"
CA35340	Papahadjopoulos, P. D.	"Liposome-Mediated Intracellular Delivery In Vitro"
CA42065	Regen, S. L.	"New Synthetic Carriers for Antitumor Drugs"
TUMOR VACCINES		
Preclinical		
CA29592	Kahan, B. D.	"Active Specific Immunotherapy in Man: A murine model"
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity by Cyclophosphamide"
CA40352	Livingston, P. O.	"Immunization of Melanoma Patients with Gangliosides"
CA36233	Mitchell, M. S.	"Specific Active Immunotherapy of Human Melanoma"
LYMPHOKINES OTHER THAN IL-2		
Interferons - Preclinical		
CA44262	Esteban, M.	"Mechanisms of Action of 2Interferon"
CA26475	Fleischmann, W. R.	"Modulation of the Anti-Tumor Effect of Interferon"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LYMPHOKINES OTHER THAN IL-2		
Interferons - Preclinical (Continued)		
CA39039	Friedman, R. M.	"A Mechanism of Action of Interferon"
CA38661	Rubin, B. R.	"Interferons-Properties, Action and Patient Prescreening"
CA43201	Ozer, H.	"Immunoregulatory Effects of the Interferons"
Interferons - Clinical		
CA45814	McGlave, L. B.	"Regulation of Hematopoiesis in CML by Gamma Interferon"
CA26966	Meyers, J. D.	"Human Leukocyte Interferon After Marrow Transplant"
Interleukin-1 - Preclinical		
CA38043	Lachman, L. B.	"Biologic Studies of Human Interleukin-1"
CA45143	Gaffney, E. V.	"Malignant Cell Growth Modulation by Interleukin-1"
CA46259	Gallicchio, V. S.	"Control of Hematopoietic Toxicity With Interleukin-1"
CA48077	Johnson, C. S.	"Cytokine Enhancement of Chemotherapeutic Efficacy"
Tumor Necrosis Factor - Preclinical		
CA44365	Sehgal, P. B.	"Tumor Necrosis Factor Induces a New Regulatory Cytokine"
B-Cell Cytokines - Preclinical		
CA42006	Choi, Y. S.	"Human B-Cell Growth Factor"
CA45148	Maizel, A. L.	"Biomolecular Nature of Human B-Cell Maturation Factor"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LYMPHOKINES OTHER THAN IL-2		
B-Cell Cytokines - Preclinical (Continued)		
CA35845	Sidman, C. L.	"Purification and Immunobiology of B-cell Maturation Factors"
Other Lymphokines - Preclinical		
CA45672	Lilly, M.	"Therapeutic Uses for Human Granulocyte CSF"
CA40567	Schnaper, H.W.	"Inhibition of Tumor Cell Growth by the Lymphokine SIRS"
LAK +/- IL-2		
Preclinical		
CA38842	Pauly, J. L.	"Interleukin-2 Mediated Regression of Breast Tumor"
CA47097	Ochoa, A. C.	"Long-term LAK Cells: Effectors and In Vivo Relevance"
Clinical		
CA39489	Mier, J. W.	"In Vivo Effects of Recombinant Human Interleukin"
CA43950	Mier, J. W.	"Immunobiology of LAK Cells"
CA43977	Stenzel, K. H.	"Renal Cell Carcinoma: Adoptive Immunotherapy"
BCG		
Preclinical		
CA15325	Gray, G. R.	"Anti-Tumor Active Components of BCG Cell Walls

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
AUTOLOGOUS SPECIFIC ANTI-TUMOR CYTOXIC CELLS		
Preclinical		
CA48075	Bear, H.	"Expansion of Anti-Tumor T-Cells From Tumor-Bearing Host"
CA30558	Cheever, M. A.	"Specific Immunotherapy of Murine Tumors"
CA39286	Mastrangelo, M. J.	"Developing T-cell Clones Cytoxic to Sarcomas"
CA48109	Roberts, L. K.	"Suppressor T-Cell Manipulation as a Cancer Immunotherapy"
CA49231	Shu, S.	"Adoptive T-cell Immunotherapy of Nonimmunogenic Tumors"
Clinical		
CA41741	Braun, D. P.	"Arachidonic Metabolism in Cancer Patient Macrophages"
CA43244	Kradin, R. L.	"Clinical Use of T-Lymphocytes for Therapy of Lung Cancer"
TUMOR ANTIGENS		
Preclinical		
CA33049	Oettgen, H. F.	"Monoclonal Antibodies in the Treatment of Cancer"
CA42508	Reisfeld, R. A.	"Molecular Profile of Melanoma and Neuroblastoma Antigens"
INVESTIGATIONS OF MATURATION OR DIFFERENTIATION INDUCERS		
Preclinical		
CA35999	Chiao, J. W.	"Maturation Induction of Human Leukemic Cells"
CA42006	Choi, Y. S.	"Human B-cell Growth Factors"
CA17393	Donahoe, P. K.	"Muellerian Inhibiting Substance"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
INVESTIGATIONS OF MATURATION OR DIFFERENTIATION INDUCERS		
Preclinical - (Continued)		
CA45783	Friedman, E. A.	"Malignant to Benign Tumor Transition"
CA38627	Sonenberg, M.	"Growth Hormone and Differentiation"
CA47589	Santoli, D.	"Growth Factor-Dependent Normal and Leukemic Cell Growth"
BONE MARROW TRANSPLANTATION		
Preclinical		
CA43026	Stewart, F. M.	"Alternative Sources of Hematopoietic Renewal"
Clinical		
CA23766	O'Reilly, R. J.	"Marrow Transplantation in Leukemia and Blood Diseases"
CA18221	Storb, R. F.	"Marrow Grafting for Treatment of Hematologic Malignancies"
CA36725	Vallera, D. A.	"Immunotoxins in Human Bone Marrow Transplantation"
METASTASIS		
Preclinical		
CA30726	Niederkorn, J. Y.	"Immunologic Modulation of Ocular Tumor Metastasis"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
TOXINS		
Preclinical		
CA42354	Maxwell, I. H.	"Tumor Cell Killing by Expression of a Toxin Gene"
CA4146	Murphy, J. R.	"Diptheria Toxin Related Peptide Hormone Gene Fusions"
SBIR		
Phase I		
CA48573	Hirschel, M.	"Automated Cultue of Lymphokine Activated Cells"
Phase II		
CA39942	Mix, T. W.	"A New Large Scale Affinity Separation Technique"
CA44246	Sobol, R. E.	"Monoclonal Antibody Anti-Idiotype Tumor Vaccines"
OTHER TOPICS		
Preclinical		
CA47324	Cox, D. C.	"Murine Tumor Immunotherapy Using Non-infectious Reovirus"
IMMUNOTOXINS		
Leukemia - Preclinical		
CA45568	Taetle, R.	"Immunotoxins for Treatment of Leukemia and Lymphoma"
CA48068	Ramakrishnan, S.	"Inhibition of Tumor Cell Growth By Immunotoxins"
CA49254	Raso, V. A.	"Model to Test the Therapeutic Value of Toxin Conjugates"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
CONFERENCE		
CA47337	Lachman, L. B.	"Sixth International Lymphokine Workshop"
CA48097	Royston, I.	"Third International Conference on Monoclonal Antibodies"
CA49974	Colby, C. B.	"Society for Biological Therapy Annual Meeting"

TABLE III

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

Institutions Currently Holding Phase I/II Clinical Task Orders

Task A: Phase I Clinical Studies of Monoclonal Immunoconjugates in Cancer Patients

Memorial Sloan-Kettering Institute for Cancer Research
University of Alabama
University of Texas, M.D. Anderson Cancer Center

Task B: Phase I/II Clinical Trials of Biological Response Modifiers Clinical Studies of Cytokines and Immunomodulators

Cleveland Clinic
Memorial Sloan Kettering Institute for Cancer Research
University of Southern California, Los Angeles
University of Wisconsin

TABLE IV

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

PHASE I CLINICAL TRIALS FUNDED BY MASTER AGREEMENT ORDERS

Institution	Agent
Georgetown University	Leukocyte Interferon*
Sidney Farber Institute	Leukocyte Interferon*
Northern California Cancer Program	Leukocyte Interferon*
University of California, Los Angeles	Lymphoblastoid Interferon*
Duke University	Lymphoblastoid Interferon*
University of Wisconsin	Lymphoblastoid Interferon
University of California, San Diego	Thymosin*
Fred Hutchinson Cancer Research Center	Thymosin*
George Washington University	Thymosin*
Sloan-Kettering Institute	Thymosin*
Northern California Cancer Program	Thymosin*
Vanderbilt University	MVE-2*
Ohio State University	MVE-2*
University of California, Los Angeles	Anti-T Cell Monoclonal Antibody*
University of California, San Diego	Anti-T Cell Monoclonal Antibody*
University of Southern California	Anti-T Cell Monoclonal Antibody*
Fox Chase Cancer Center (Jefferson Medical College)	Anti-T Suppressor Cell Antibody*
Pittsburgh Cancer Center	Anti-lymphoma Monoclonal Antibody and IL-2
Ohio State University	rInterferon-beta and gamma*
University of California, Los Angeles	rInterferon-beta and gamma*
University of Southern California	rInterferon-beta*
University of Wisconsin	rInterferon-gamma*
Duke University	rInterferon-gamma*
University of Wisconsin	rIFN-beta and rIFN-gamma - 2 trials
Yale University (University of Pittsburgh)	rIFN-alpha and rIFN-gamma
Illinois Cancer Council	Anti-T Cell Monoclonal Antibody Radio-Labeled Conjugate
University of California, San Diego	Anti-T Cell Monoclonal Antibody Conjugate*
Fred Hutchinson Cancer Research Center	Anti-melanoma Monoclonal Antibody and IL-2
Memorial Sloan Kettering Cancer Center	$F(ab')_2$ Fragments of Anti-melanoma Monoclonal Antibody
Memorial Sloan Kettering Cancer Center	Anti-melanoma Monoclonal Antibody and IL-2
Cleveland Clinic	Anti-melanoma Monoclonal Antibody and r-IFN-a*
Cleveland Clinic	Anti-melanoma Monoclonal Antibody and DTIC

TABLE IV (Continued)

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

PHASE I CLINICAL TRIALS FUNDED BY MASTER AGREEMENT ORDERS

Institution	Agent
University of Wisconsin	Anti-ovarian Monoclonal Antibody and rIFN-gamma
University of Southern California	Anti-lymphoma Monoclonal Antibody and rIL-2
University of Alabama at Birmingham	Anti-melanoma Monoclonal Antibody and rIL-2
University of Texas M.D. Anderson Can. Ctr.	Anti-melanoma Monoclonal Antibody and IFN-alpha
Mt. Sinai Cancer Center	Anti-lymphoma Monoclonal Antibody and rIL-2
University of Wisconsin	IL-2 (Recombinant) - 2 trials*
Illinois Cancer Council	IL-2 (Recombinant) - 2 trials
University of Wisconsin	LAK cells and Cyclophosphamide
	LAK cells and Cyclophosphamide and IL-2
Ohio State University	IL-2 and LAK Cells
University of Wisconsin	rIL-2 and LAK Cells
Cleveland Clinic	rIL-2 and TIL
University of California, Los Angeles	rIL-2 and Thoracic Duct Lymphocytes
Pittsburgh Cancer Center	rIL-2 and LAK Cells
Thomas Jefferson University	rIL-2 and Cytotoxic T-Cells
Cleveland Clinic	GM-CSF-Activation of Macrophage
University of California, Los Angeles	GM-CSF-Therapeutic Effects of IV vs Sub Q
New York University	GM-CSF-Macrophage Activation

PHASE II CLINICAL TRIALS FUNDED BY MASTER AGREEMENT ORDERS

Institution	Agent
Georgetown University	Lymphoblastoid Interferon*
University of California, Los Angeles	Lymphoblastoid Interferon*
Duke University	Lymphoblastoid Interferon*
Sloan-Kettering Institute	Lymphoblastoid Interferon*

* Completed trials

TABLE V

BIOLOGICAL RESPONSE MODIFIERS PROGRAM
BIOLOGICAL RESOURCES BRANCH
CONTRACTS PROJECTED FOR FUNDING IN FY 89

CONTRACTS	Estimates ($K)
Preclinical	
Chemical Coupling of Cytotoxic Agents to Tumor Reactive Monoclonal Antibodies	$ 30
Collection, Storage, Distribution, and Quality Assurance of BRM	425
Animal Lymphokine Evaluation	189
Large Scale Production of MoAbs	659
Animal Production	300
Monoclonal Antibody Purchases	382
Clinical	
Master Agreement Orders	389
Phase I Contracts	
Task A: Phase I Clinical Trials of Monoclonal Antibodies	1,054
Task B: Phase I Clinical Trials of Cytokines	1,733
Total	$5,161

SUMMARY REPORT

LABORATORY OF MOLECULAR IMMUNOREGULATION

October 1, 1988 through September 30, 1989

INTRODUCTION

Headed by Dr. Joost J. Oppenheim, it is the mission of the Laboratory of Molecular Immunoregulation (LMI) to investigate at the cellular, biochemical, and molecular level, the intercellular and intracellular processes that regulate host defense mechanisms, including: interaction of cytokines with specific cell surface receptors; the membrane, cytoplasmic, and nuclear events which occur after interaction of these molecules with their receptors; the lymphokine/ cytokine modulation of cellular functions that participate in host defense; the effects of Biological Response Modifiers (BRMs) on immunoregulatory pathways and the development of new BRMs that enhance host defense mechanisms; and the role of immunoregulatory pathways in the pathophysiology of diseases. The LMI officially consists of three sections: Immunobiology, Lymphokine, and the newly formed Cytokine Molecular Mechanisms Section (CMMS) that was founded at the beginning of 1989. In addition, a fourth research group in the LMI that concentrates on studies of inflammatory cytokines, operates through the Office of the Chief.

RESEARCH MANAGED THROUGH THE OFFICE OF THE CHIEF

Three of the LMI investigators, Kouji Matsushima, M.D., Ph.D., Joost J. Oppenheim, M.D. and Scott K. Durum, Ph.D., are engaged predominantly in research studies concerning the contribution of inflammatory cytokines to host defense.

Dr. Oppenheim, in collaboration with Ruth Neta, Ph.D., Section of Experimental Hematology of the Armed Forces Radiation Research Institute, observed that pretreatment with interleukin 1 (IL 1) protects mice from lethal doses of irradiation. This collaborative study of the in vivo effects of these cytokines has been extended to show beneficial effects of a number of different cytokines, the effect of therapy with cytokines following irradiation, and the beneficial effects of combining cytokine treatment with bone marrow grafts. IL 1 was shown to synergistically enhance the survival of lethally irradiated mice who were also administered syngeneic or allogeneic bone marrow cells. Mice that survived over six weeks following treatment with allogeneic bone marrow cells and IL 1 were mixed chimeras and exhibited tolerance to major histocompatibility antigens of the class II type of both host and donor origin. These data suggest that IL 1 may prove useful in treatment of patients undergoing bone marrow transplantation. A number of other investigators, including Dan L. Longo, M.D., and Ron Gress, Ph.D., of the Immunology Branch, NCI, and Robert Wiltrout, Ph.D. of the LEI, have collaborated on various aspects of this study.

Dr. Matsushima is at present a Visiting Scientist who heads a group consisting of two postdoctoral fellows and two guest researchers that are engaged in investigating the production and activities of IL 1 at the molecular and biochemical level. This group is studying the mechanism of action of IL 1 by studying the receptor mediated signal transduction events induced by IL 1. They have observed that IL 1 and also tumor necrosis factor rapidly

phosphorylate two intracellular proteins, p65 which has been purified, sequenced and identified to be l-plastin and a p74. The serine residues of the proteins are phosphorylated by an as yet unidentified serine kinase which is distinct from protein kinase C, protein kinase A or casein kinase.

Dr. Matsushima together with one postdoctoral fellow and six Guest Researchers have also pursued studies of the newly discovered human cytokine which is chemotactic for neutrophils. They have observed that this cytokine is produced not only by monocytes but also by T lymphocytes, fibroblasts, endothelial cells and keratinocytes. This chemotactic factor also is an in vitro chemoattractant for T lymphocytes and results in the rerouting and accumulation of lymphocytes in draining lymph nodes. The cytokine has been purified, sequenced and genetically cloned by Dr. Matsushima and coworkers. Since this cytokine acts on several major inflammatory cell types, his laboratory proposed that it be renamed interleukin 8 (IL 8). Dr. Matsushima and Dr. Yamada, our collaborator at the Dainippon Pharmaceutical Company, have succeeded in expressing recombinant human IL 8 in quantity from E.coli which has enabled us to make recombinant IL 8 available to many research laboratories upon request. Patent applications have been submitted regarding the structural features and functional activities of IL 8.

Dr. Matsushima and his colleagues have also indentified and purified another novel cytokine which selectively chemoattracts monocytes and activates them to inhibit tumor cell proliferation. This cytokine has been named MCAF and shows significant amino acid homology to IL 8. MCAF has been genetically cloned by our collaborator Dr. Yamada. Dr. Matsushima and coworkers are presently studying the structure, function relationships of MCAF and the receptors for IL 8 and MCAF. Patent applications have also been submitted for MCAF. Dr. Matsushima's biochemical expertise has provided the LMI with the requisite expertise to purify, identify and genetically clone new cytokines.

Dr. Durum is a Senior Staff Fellow who currently supervises the training of two postdoctoral fellows and works with two guest investigators on the problem of the contribution of cytokines such as IL 1 to the immune response. One important effect of IL 1 has been the co-stimulation with antigen of interleukin 2 (IL 2), a lymphocyte growth factor. Dr. Durum and coworkers have determined the pathway by which IL 1 and antigens activate the promoter on the IL 2 gene. They have demonstrated that IL 1 induces increased mRNA for "jun", whereas stimulation of the antigen receptor (TcR) induces "fos" mRNA. The jun and fos proteins interact and lead to the formation of the AP-1 transcription factor which in turn binds to and activates the promoter on the IL 2 gene. Induction of AP-1 may reflect a more general pathway of IL 1 action, since induction of jun has also been detected in IL 1 stimulated glioblastoma and hepatoma cell line cells.

Dr. Durum and colleagues are also studying the role of cytokines in T cell development. They have shown that isolated and purified immature "double-negative" mouse or rat thymocytes when stimulated with IL 1 will proliferate and develop into more mature differentiated CD4+, TcR α/β expressing thymocytes. In contrast, incubation of double-negative thymocytes with IL 2 leads to proliferation and development of more mature CD8+ TcR γ/δ expressing thymocytes with cytotoxic activities. These in vitro effects may reflect in vivo pathways of thymocyte development. Dr. Durum serves as the LMI expert on issues involving the role of cytokines in immune mediated host defense.

IMMUNOBIOLOGY SECTION

The Immunobiology Section conducts studies on the intracellular events responsible for the differentiation of lymphocytic and myelocytic cells and their activation to exert antitumor effector functions; studies the structure-function relationship between RNA secondary structures, transport stability and gene expression; conducts studies on the changes in cell surface and intracellular markers during the process of cell differentiation, activation, and lymphokine receptor interaction; investigates the role of cytokines, such as interferon, colony stimulating factor (CSF), IL 1 and of oncogenes, such as fos, ras, myc and raf, in cell growth and differentiation; and analyzes the role of DNA structure in the control of lymphokine gene expression.

Luigi Varesio, Ph.D., the Acting Head of the Immunobiology Section together with two postdoctoral scientists and three guest investigators have been focusing their efforts on the molecular basis for macrophage growth and differentiation and the acquisition of antitumor activity. In order to facilitate these studies, Dr. Varesio and coworkers have established immortalized murine macrophage cell lines by transfecting normal bone marrow cells with recombinant retroviruses expressing both the oncogenes v-myc and v-raf, but not with the individual v-onc molecules. This has provided Dr. Varesio with homogeneous populations of functional macrophages for studies of intracellular events following macrophage activation by various ligands. Dr. Varesio has shown that the functional response of the macrophage lines to cytokines was intact. Moreover, he has observed that the expression of the transforming viral genome could be inhibited by picolinic acid and IFNγ by decreasing the stability of the viral mRNA. These results indicate that post-transcriptional events may be important in controlling viral gene expression in macrophages.

Dr. Varesio and coworkers have investigated the molecular events associated with the expression of cytotoxicity by macrophages. They have successfully demonstrated the existence of different mechanisms involved in the activation of human monocytes by IL 2 versus IFNγ since IL 2, but not IFNγ requires active protein kinase C. Moreover, activation of monocytes by IL 2 and not by IFNγ is associated with an increase in colony stimulating factor 1 (CSF1) receptor. Exogenous CSF1 synergized with IL 2 and not IFNγ in the activation of cytotoxic human monocytes. The β as well as the α chain of the IL 2 receptor is constitutively expressed on human monocytes. Overall, these results indicate that the interaction of IL 2 with the β chain is sufficient to trigger the cytolytic activity and the expression of CSF1 receptor, and that CSF1 can further augment the activation of IL 2-activated monocytes. In contrast, IL 2 does not activate mouse macrophages or augments CSF1 receptor expression, although it synergizes with IFNγ. The triggering the α chain of the IL 2 receptor by IFNγ is needed for the response of these cells to IL 2.

Studies have also been performed by Dr. Varesio and colleagues on the expression of human immunodeficiency virus-long terminal repeat (HIV-LTR) binding protein during macrophage activation. Macrophages constitutively express nuclear proteins binding to HIV-LTR. Moreover, activation of macrophages by endotoxins induces the synthesis of a new protein involved with HIV-LTR binding activity. These results provide the first evidence that the expression of HIV-LTR binding proteins can be modulated during the activation process and suggest that such protein may be important in regulating the expression of HIV in macrophages. Consequently, Dr. Varesio provides the LMI with expertise in studies of the role

of cytokines and intracellular mechanisms in activating macrophages to participate in host defense.

LYMPHOKINE SECTION

The Lymphokine Section investigates the mechanism of action at the biochemical and molecular level; the therapeutic usefulness of lymphokines and other lymphocyte-derived growth and differentiation factors in the treatment of cancer; monitors the effects of treatment with lymphokines on immune responses; and investigates the role of oncogenes and proto-oncogenes in the modulation and alteration of immune and hematopoietic system function and development.

Francis W. Ruscetti, Ph.D., Head of the Lymphokine Section, supervises a group consisting of three postdoctoral fellows and one guest investigator. Dr. Ruscetti's research focuses on the effect of cytokines such as transforming growth factor β (TGF β), IL 2, IL 3 and other CSF on the growth and differentiation of normal and neoplastic hematopoietic cells. Of particular interest are studies showing that TGF β is a bi-functional regulator of hematopoietic cell growth, and that TGF β1 and TGF β2 are equipotent inhibitors. TGF β has a selective effect on hematopoiesis by inhibiting the growth of early human and murine progenitors, but not more differentiated progenitors. Using purified murine hematopoietic stem cells in single cell assays with IL 3, it was shown that TGF β acted directly on the cells to block growth. The earliest measurable events in vitro such as high proliferative potential colony formation (HPP-CFU) and induction of Thy-1 antigen were also inhibited by TGF β. In erythroid development, CFU-GEMM and BFU-E, but not GFU-E, were inhibited. Thus, the ability of TGF β to block hematopoietic cell growth depends on the differentiated state of the cell. In contrast, addition of TGF β together with murine GM-CSF greatly augmented bone marrow cell growth. This increase was primarily in the granulocyte compartment. Bone marrow cells 48 hours after 5-fluorouracil marrow treatment contain a progenitor cell whose growth absolutely depended on GM-CSF and TGF β. In vivo experiments with TGF β show the same effects on hematopoiesis as in vitro. In contrast to myeloid leukemic cells and normal lymphoid cells which were sensitive to TGF β inhibition, cultured lymphoid leukemic cells were insensitive to TGF β. Functional TGF β receptors leading to growth inhibition could be induced on these leukemic cells by phorbol myristate acetate (PMA). Thus, the loss of negative regulation by TGF β may play a role in the growth of some leukemic and malignant lymphoid cells. The possible in vivo therapeutic effects of TGF β on leukemic and normal cell growth are being evaluated.

Dr. Ruscetti's other major research interest centers on the interactions between human retroviruses such as HTLV I, II and III (HIV-I) and leukocytes. Since these retroviruses are all associated with chronic, slowly progressive diseases, Dr. Ruscetti investigated whether host-cell restriction of viral expression, resulting in little or no viral production, is involved in pathophysiology of these diseases. Dr. Ruscetti has developed in vitro cell line models to study latency of both HTLV-I and HIV-I. In HTLV-I infected B cells from acute T cell leukemia (ATL) patients, no viral mRNAs are being transcribed, yet integrated provirus is functional and can be activated to transform other T and B cells. When the virus infects T-cells, the virus is expressed; when it infects B cells, it is not expressed. Also, the macrophage, a reservoir of HIV-I infection in AIDS patients, was shown to be able to restrict HIV-I expression. THP-1, a macrophage cell line, was permissive for HIV-I expression. However, these

infected cells become naturally non-productive for HIV four to six weeks after infection. Two classes of restricted HIV-I expression were seen: 1) low-level which can be regulated by factors in the nuclei of infected cells; and 2) no expression which may be due to methylation of the long terminal repeat (LTR). Both viruses make proteins which act in trans to positively regulate viral transcription. In these latently infected cultures, transactivation of viral transcription is inhibited. This indicates that the host factors play a role in this transcription and these cells either are deficient in positive regulators or possess negative regulators of viral transcription. Understanding the mechanisms of action of negative regulators of viral expression can be useful in developing anti-viral therapies. As indicated above, Dr. Ruscetti serves as the local LMI and BRMP expert in studies of hematopoiesis, leukemia, human retroviruses, and AIDS.

CYTOKINE MOLECULAR MECHANISMS SECTION

The Cytokine Molecular Mechanisms Section (CMMS) was established based on the fact that investigators in the Lymphokine Section were pursuing conceptually and experimentally distinct goals. The new CMMS was formed during the fiscal year. The Section is headed by William L. Farrar, Ph.D., and has two distinct missions:

1. Characterization of common biochemical pathways between the immune and central nervous systems at the biochemical, molecular and biological levels. The identification of signal peptides (cytokines and neuroendocrine hormones), their recognition sites (receptors), and biological responses in tissues derived from both immune and neurologic sources. The studies will also include the examination of the expression of mRNA specific for both regulatory peptide signals, their receptors, and the genetic expressions elicited by the biological peptides.

2. Characterization of interaction of HTLV III/LAV retrovirus with cells of neuronal and immune origins. The pathobiology of virus in neuronal cell lines will be studied in vitro, as well as the effects of virus pathology on brain neuroreceptor function in brain sections from both primate and human specimen. The section will continue its development of synthetic peptides which antagonize HTLV III/LAV envelope protein binding and exhibit antiviral activity in immunological tissues.

Dr. Farrar together with two postdoctoral trainees and several guest investigators is studying cytokine receptor signal transduction, and the regulation of HIV expression by cytokines. Cytokine receptor signal transduction is examined by isolating the protein kinases associated with the specific receptor. This laboratory has recently shown that hematopoietic cytokines stimulate one or more tyrosine kinases. The receptors for these cytokines lack intrinsic kinase domains but apparently couple to these protein kinases. They are characterizing the substrates of these tyrosine kinases and are examining homologous cell lines transformed with v-src family oncogenes for substrates commonly phosphorylated by growth factor stimulation and tyrosine kinase oncogenes.

The Section has studied the genetic basis of lymphohematopoietic cell proliferation by analysis of the genes expressed during the cytokine regulated cell cycle and the activation of "enhancer" DNA binding proteins. IL 2

regulates the transcription of a number of genes including the proto-oncogenes, c-fos, c-myc, c-myb as well as other genes such as ornithine decarboxylase (ODC) and the IL 2 receptor α chain (Tac). Antiproliferative signals such as cyclic AMP selectively inhibit IL 2 directed increases in c-myc and ODC mRNA accumulation. The specific deletion of c-myc protein biosynthesis by synthetic "anti-sense" oligonucleotides inhibited PHA or IL 2 directed proliferation of human T cells. IL 2 also stimulates mRNA expression and protein synthesis of ancient stress genes such as HSP-70. The Section has investigated the DNA binding proteins which recognize the enhancer regulatory element of the HSP-70 gene. They are likewise examining DNA binding proteins specific for the c-myc and IL 2 receptor and HIV-LTR enhancer sequences to determine what signals modulate the effects of these proteins on gene transcription.

Dr. Farrar and coworkers have also examined the relationship of HIV infection on the release of cytokines and their subsequent autocrine effects on HIV replication. The HIV envelope protein (gp120) was shown to be a potent stimulator of monocyte function, causing the release of IL 1 and PGE2. Additionally, gp120 alters human monocyte chemotaxis and inflammatory cell function. Mediators released by monocytes, such as tumor necrosis factor (TNF) increased HIV productivity in latently infected T cell lines. Thus, Dr. Farrar provides the LMI and BRMP with expertise in studies of neuroimmune interactions and post-receptor intracellular signal transduction.

INTERRELATIONSHIPS BETWEEN LMI INVESTIGATORS

The research programs of each of the individual LMI investigators are thematically interrelated. Although each of the investigators is studying various aspects of the properties and activities of cytokines, there is little or no overlap in their specific areas of interest. For example, Dr. Matsushima's work focuses on the biochemical and molecular aspects of human cytokines, whereas Dr. Durum asks questions concerning the immunological role of these cytokines using in vitro and in vivo murine models. Dr. Varesio is primarily interested in molecular studies of monocyte/macrophages. Dr. Farrar provides other LMI investigators with expertise concerning cytokine induced signal transduction pathways, gene regulation, and neuro-immune pathways. Finally, Dr. Ruscetti is the LMI expert on cytokines affecting normal and neoplastic hematopoietic cells and on the interaction of retroviruses such as HTLV 1 and HIV 1 with such cells.

Each of the LMI investigators hold weekly work seminars. All LMI investigators also meet on a weekly basis to review the results and plans of an ongoing study, report on scientific meetings or learn from an invited lecturer. In addition, there are weekly BRMP-wide invited lecturers, staff meetings, yearly conferences and casual contacts at which LMI investigators have the opportunity to exchange information. As is evident from LMI publications, there is considerable collaboration of LMI investigators with one another, as well as with other BRMP, NCI, NIH, and non-NIH scientists. The monthly therapeutic application seminars held in conjunction with the clinical investigators of the Clinical Research Branch (CRB), BRMP provide additional impetus and fruitful interactions for the LMI scientists. This provides a forum which enables LMI scientists to contribute to formulating protocols for clinical evaluation of biologicals. There are also many mutually beneficial consultative contacts and interchanges between LMI scientists and the extramural scientist administrators in the Biological Resources Branch (BRB), BRMP. This facilitates our ability to obtain

recombinant cytokines from biotechnology firms and foster collaborative studies and information exchange with the extramural community. Finally, LMI scientists not only participate in many meetings with extramural scientists, but also have engaged in co-organizing several cytokine meetings such as the upcoming Second International Cytokine Workshop this fall.

Early in the fiscal year, Howard A. Young, Ph.D., left the LMI and accepted a promotion to the position of Section Head in the Laboratory of Experimental Immunology of the BRMP. We have been evaluating numerous candidates to take Dr. Young's FTE position as a molecular biologist with an immunology background in the LMI. Although Dr. Young is certainly still readily available to the LMI for consultation and collaborative studies, we plan to further strengthen the expertise in molecular approaches of the LMI with which to study the inter- and intra-cellular signals that mediate host defense to cancer and AIDS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09289-04 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Restorative Role of Cytokines in Hematopoiesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. J. Oppenheim	Chief	LMI, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	R. Gress	Senior Investigator	C, NCI
	D. Sachs	Senior Investigator	C, NCI

COOPERATING UNITS *(if any)*

Armed Forces Radiobiology Research Institute, Bethesda, MD (R. Neta); PRI, NCI-FCRF (P. Tiberghien, R. Hornung, J. Kenny)

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Prior administration of cytokines, especially IL 1 can protect mice from lethal doses of irradiation ranging from 900 to 1,200 cGy. Cytokines, if administered in a therapeutic mode after irradiation can protect mice up to doses of 1,000 cGy. A number of experimental approaches showed that these beneficial effects of the cytokines were based on the recovery of suppressed hematopoietic tissues. This was substantiated by experiments showing that co-administration of IL 1 along with 10 to the sixth to 10 to the seventh syngeneic bone marrow cells synergisticially promoted the survival of mice irradiated with up to 1,500 cGy.

IL 1 also enhanced the capacity of allogeneic bone marrow cells to promote survival of mice given doses of radiation ranging from (1,100-1,350 cGy). Three to five times greater numbers of lethally irradiated (1,200-1,350 cGy) C57Bl/6 (H-2b) mice given 10 to the seventh T-cell-depleted Balb/c (H-2d) BM cells survived >6 weeks if also treated with a single IP dose of 10 μg IL 1 20 hours after radiation. The spleens of these mice were reconstituted predominantly but not exclusively (54-91%) with donor cells. Spleen cells from such allotransplanted IL 1 treated mice that survived from 6 to 12 weeks were able to generate specific T cell cytotoxic killing of unrelated allogeneic donor cells, but were unreactive to target cells bearing either host or donor MHC class I antigens. Thus, long term survivors were chimeric and tolerant to recipient and donor alloantigens, but exhibited immunological competence. These results show that IL 1 promotes survival of lethally irradiated mice and that allogeneic hematopoietic cells in such animals develop tolerance to host MHC antigens. Although there are many unanswered questions, these data suggest that IL 1 may prove clinically useful in patients undergoing bone marrow transplantation.

PHS 6040 (Rev 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Joost J. Oppenheim	Chief	OC, LMI, NCI
Dan L. Longo	Associate Director	OAD, NCI
Ron Gress	Senior Investigator	C, NCI
David Sachs	Senior Investigator	C, NCI

MAJOR FINDINGS

The purpose of this study focuses on the protective and therapeutic effects of cytokines in counteracting the deleterious effects of irradiation and chemotherapy in animal models. Protective effects mean effects achieved by pretreatment with cytokines which increases the tolerance of an animal to radiation or chemotherapy. On the other hand, therapeutic effects of cytokines mean effects obtained by treatment after radiation or chemotherapy which are presumably based on induction of restorative repair and recovery mechanisms.

Radioprotective Effects of Cytokines

We have observed that prior administration of IL 1 and TNF, but not G-CSF, IL 2 or IFN α,β have radioprotective effects. When administered together, IL 1 and TNF have additive or synergistic radioprotective effects suggesting that their mechanisms of action differ. This observation argues against IL 1 mediating the radioprotective effect of TNF, or the converse. The effect of combined treatment with IL 1 and TNF was also greater than that achieved with optimal radioprotective doses of LPS, an inducer of IL 1 and TNF. This result suggests that the two cytokines can be administered at a more effective ratio, and that toxic effects of LPS itself can be circumvented by using the purified cytokines.

Although both GM-CSF and G-CSF did not protect against lethal doses of irradiation by themselves, they had synergistic radioprotective effects when administered in conjunction with suboptimal doses of IL 1. The synergistic interactions of CSF and IL 1 also suggest that these cytokines may act in concert on in vivo hematopoiesis. IL 6, like IL 1, acts as an hematopoietin in vitro and costimulates in conjunction with CSF the formation of high proliferative colonies (HPP). However, in contrast with IL 1, IL 6 is a radiosensitizer rather than radioprotective agent and IL 6 makes mice more sensitive to radiation. Unlike IL 1, IL 6 does not induce the production of CSF which may account for its failure to protect mice against radiation. Despite the inhibitory effects of TNF on in vitro hematopoietic colony formation, like IL 1, TNF induces CSF and may thus indirectly promote hematopoiesis.

The patterns of hematopoietic recovery in endotoxin treated animals closely resembles the hematopoietic recovery in animals receiving syngeneic bone marrow transplants. Consequently, the accelerated restoration of functional immunocytes and hematopoietic cells is believed to be a major factor in the survival of lethally irradiated mice. IL 1-treated, as compared to non-treated,

mice five days after irradiation had greatly enhanced numbers of nucleated bone marrow cells and increased endogenous splenic colonies suggesting that IL 1 administration may stimulate bone marrow hematopoiesis. In fact, our studies of normal mice established that intraperitoneal administration of IL 1 within 24 hrs significantly increased the percentage of large cells in S+G+M phases of the cell cycle. In addition, the capacity of BM cells from IL 1 treated mice to proliferate in cell suspension cultures in response to GM-CSF was greatly increased. The observed induction of cell cycling with IL 1 correlates with the enhanced radioprotective effect of this cytokine.

Therapeutic Effects of IL 1

We have also shown that therapeutic administration of IL 1 is effective in promoting hematopoietic recovery following mid-lethal doses of irradiation. However, much higher doses ranging from 1 to 10 μgm of IL 1 were needed to protect mice from 800-1000 irradiation as compared with the 100-500 μg doses of IL 1 that were radioprotective. The fact that IL 1 is not as effective when used after irradiation as before irradiation in treatment of lethally irradiated mice, may indicate that following irradiation a small number of surviving stem cells or progenitor cells may be required to serve as targets of IL 1. In order to model the potential radioprotective effect of IL 1 on patients receiving multiple doses of irradiation, mice were given IL 1 before, after and/or in between fractionated doses of radiation in the lethal range. Preliminary results were reassuring since all mice that received more than one dose of IL 1 survived.

Effect of IL 1 on Syngeneic Bone Marrow Transplants

Mice have been exposed to doses ranging from 1,250 to 2,000 r irradiation and treated with IL 1 along with isologous bone marrow cells and the duration of survival determined. Mice are synergistically protected by BM cells and IL 1 against doses as high as 1,500 r but not 2,000 r, suggesting GI toxicity may not be diminished by IL 1. Doses of isologous bone marrow cells ranging from 0.5 x 10 to the sixth to 10 to the seventh when given together with IL 1 immediately after 1,500 irradiation result in greater long-term survival than if given without IL 1. When given together with IL 1, a reduced number of 10 to the sixth isologous bone marrow cells resulted in optimal survival. Preliminary experiments in which donor bone marrow cells were incubated with 1 μg IL 1/ml for 6 hours ex vivo and then washed free of IL 1 before administration to irradiated mice did not show the survival of recipient mice. This data suggests that the effects of IL 1 are indirect and may be mediated by stimulation of hematopoietic factor production by the recipient.

The Effect of Cytokines on Bone Marrow Allografts

The effect of cytokines on allogeneic bone marrow transplants is also being studied in the mouse model. C57B16 mice immediately after irradiation with 1,100 - 1,350 r have been given various doses of allogeneic (Balb/C) bone marrow (T cell depleted) cells intravenously with or without 10 μg of IL 1.

The results clearly show that IL 1 markedly enhanced the survival of lethally irradiated mice treated with allogeneic bone marrow cells. IL 1 promoted

survival of C57Bl/6 (H-2^b) mice irradiated with 1,200-1,350 cGy and reconstituted with T cell depleted Balb/c (H-2^b) allogeneic cells despite major differences in MHC. The numbers of long term surviving mice (>1.5 mos) following treatment with 5 x 10 to the sixth or 10 to the seventh allogeneic bone marrow cells plus IL 1 far exceeded the numbers of surviving mice given only bone marrow cells. This finding suggests that IL 1 may promote either the recovery of host bone marrow cells or accelerate the engraftment and proliferation of donor bone marrow cells or both.

The dose of radiation and dose of allogeneic cells used markedly influenced the degree of engraftment by allogeneic bone marrow cells. In a preliminary study C57Bl/6 mice irradiated with 1,100 cGy were given 2 μg IL 1β intraperitoneally daily for 6 days beginning right after irradiation with or without 5-10 to the sixth partially T-cell-depleted Balb/c bone marrow cells immediately after radiation. Lethally irradiated mice with or without IL 1β therapy all died within 12-15 days, whereas 10% of the mice given allogeneic bone marrow cells without IL 1 survived for more than 6 weeks. However, 40% of mice given both bone marrow cells and IL 1 survived for more than 6 weeks. By flow cytometry the spleens of three of five long-term surviving mice still contained >95% cells of host (H-2^b) origin, one contained 43% H-2^d and 49% host cells and only one was reconstituted predominantly (78%) with donor (Balb/c)-derived cells. The spleens were small and yielded only 5-16.5 x 10^6 cells. We speculated that in this study the allogeneic cells transiently promoted survival until replaced by host's own bone marrow cells. This pilot study encouraged us to use higher doses of irradiation, a higher dose of IL 1 and more bone marrow cells in order to decrease the likelihood of host cell recovery and improve the probability of donor cell engraftment. In fact, our subsequent results show much greater engraftment by donor allogeneic bone marrow cells provided IL 1-treated mice that were lethally irradiated with 1,200 or 1,350 cGy were also given at least 5 x 10^6 bone marrow cells.

As in our previous reports, greater radioprotection was obtained by treating mice with IL 1 before rather than after 1,200 cGy doses of irradiation. However, radioprotection both by prior treatment with IL 1 without bone marrow cells was completely ineffective as was IL 1 if given by itself after 1,350 cGy irradiation. The failure of pretreatment is probably due to the high radiation dose exceeding the protective capacity of IL 1. The failure of IL 1 treatment after radiation is presumably because there no longer were a sufficient number of residual host hematopoietic stem cells and progenitor cells for the IL 1 to act on. Furthermore, few of the mice given only bone marrow cells survived 1,350 cGy. Only the combination of IL 1 given either before or after irradiation with 10 to the seventh bone marrow cells was protective against 1,350 cGy.

IL 1 pretreatment and IL 1 therapy (i.e. after radiation) were each equally effective in promoting the survival and engraftment of mice irradiated with 1,350 cGy and given 10 to the seventh allogeneic bone marrow cells. Pretreatment with IL 1 also improved the capacity of the host to support the engraftment with allogeneic cells. Thus, the beneficial effect of the preinjected IL 1 must have persisted for over 24 hours and is effective in stimulating donor bone marrow cell recovery. Even at 1,200 cGy most of the mice that were pretreated with IL 1 were reconstituted predominantly with allogeneic

donor cells. Perhaps their enhanced survival was based on a transient recovery by host cells which were then replaced by the unirradiated donor cells.

Our studies show that donor cell reconstituted spleen cells from mice surviving $\geq$ 1 1/2-5 months have the capacity to generate T cells that were cytotoxic for unrelated (H-2^k) but not donor (H-2^d) nor recipient (H-2^b) target cells. This suggests that the T cells in the chimeric mice that survive for over 6 weeks have not only become tolerant to both donor and recipient MHC antigens, but are also immunologically competent as judged by their ability to muster a specific CTL response against an unrelated allogeneic target cell.

PROPOSED COURSE

Many other questions concerning the contribution of IL 1 to survival of lethally irradiated bone marrow engrafted mice remain unanswered. The optimal dosage, schedule, and route of administration of IL 1 need to be established in rodents as well as larger animal models. The ability of IL 1 to promote engraftment of incompletely versus completely T-cell-depleted allogeneic bone marrow cells must be compared. The hematologic recovery, immune competence and tolerance to the host of IL 1-treated donor cell-engrafted mice must be evaluated in greater detail. The capacity of IL 1 to interact with other agents, such as anti-asialo GM 1 antiserum (which suppresses natural killer activity and reduces hybrid resistance to promote bone marrow engraftment) is being studied.

SIGNIFICANCE

At a minimum, it appears that IL 1 may permit the use of higher doses of radiation therapy in the conditioning regimens used to treat malignancies currently managed by allogeneic bone marrow transplantation. In some cases, IL 1 may allow the delivery of more effective therapy without the need to reconstitute with bone marrow cells. Where allogeneic bone marrow needs to be given, IL 1 may allow full hematopoietic reconstitution with fewer donor bone marrow cells. Finally, the proper use of an appropriate mix of IL 1 and bone marrow cells may permit the establishment of hematopoietic chimerism with full immune competence, selective tolerance to donor MHC, and freedom from GVH.

PUBLICATIONS

Oppenheim JJ, Akahoshi T, Matsushima K, Tosato G. Modulation of IL 1 receptor expression by IL 1, prostaglandins and glucocorticoids. In: Mani J-C, Dornand M, eds. Proceedings of the 18th International Conference. New York: Walter de Gruyter and Co Publ, 1988;209-16.

Neta R, Oppenheim JJ. Synergistic activity of IL 1 with TNF and IL 1 with CSF in radioprotection. In: Powanda WC, Oppenheim JJ, Kluger MJ, Dinarello CA, eds. Monokines and Other Non-Lymphocytic Cytokines. New York: Alan R Liss Inc, 1988;359-64.

Powanda M, Oppenheim JJ, Kluger M, Dinarello CA, eds. Monokines and Other Non-Lymphocytic Cytokines. In: Progress in Leukocyte Biology. New York: Alan R Liss Inc 1988;1-414.

Oppenheim JJ, Larsen CG, Samanta AK, Anderson AO, Matsushima K. Biological and biochemical properties of a chemotactic cytokine. In: Petrov RV, ed. Advances in Science and Technology, 1989;43-51.

Durum S, Oppenheim JJ, Neta R. Immunophysiological role of interleukin 1. In: Oppenheim JJ, Shevach E, eds. Textbook of Immunophysiology. New York: Oxford Univ Press, in press.

Oppenheim JJ, Akahoshi T, Aiello FB, Lew W, Neta R, Matsushima K. Interactions between IL 1 and glucocorticoid hormones in inflammation. Japan J Hematol, in press.

Neta R, Oppenheim JJ, Durum SK. The cytokine concept. In: Cohen S, ed. Lymphokines and the Immune Response. New York: CRC Press, in press.

Neta R, Oppenheim JJ. Editorial: What Should Internists be Interested in Interleukin 1. Ann Intern Med, in press.

Oppenheim JJ, Shevach E, eds. Textbook of Immunophysiology. New York: Oxford Univ Press, in press.

Neta R, Oppenheim JJ. Cytokines in therapy of radiation injury, Blood, in press.

Oppenheim JJ, Matsushima K, Yoshimura T, Leonard EJ, Neta R. Relationship between IL 1, TNF and a neutrophil attracting peptide (NAP-I). Agents and Actions, in press.

Oppenheim JJ, Durum S, Larsen CG, Samanta AK, Matsushima K. Inflammatory cytokines exhibit pleiotropism, redundancies and cooperative interactions. In: Maheshwari R, ed. Cell Mediated Immunity in Relation to Tropical Diseases. Lucknow, India Press, in press.

Oppenheim JJ, Leonard EJ. Introduction: Perspective on the "Big Eaters". In: Asherson GL, Zembala M, Fullerlove G, eds. Human Monocytes, in press.

Apte RN, Oppenheim JJ, Durum SK. β-endorphin regulates interleukin 1 production and release by murine bone marrow macrophages. In: Tada T, ed. International Immunology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Basis for Macrophage Activation and Immortalization

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Varesio	Visiting Scientist	LMI, NCI
Others:	M. Clayton	Microbiologist	LMI, NCI
	G. Cox	BTP Fellowship	LMI, NCI
	P. Latham	BTP Fellowship	LMI, NCI
	I. Espinoza	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Immunobiology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies were performed to investigate the mechanisms leading to the activation of mouse macrophages and human monocytes to a cytotoxic stage. We demonstrated the existence of different mechanisms involved in the activation of human monocytes by IL 2 or IFNγ since IL 2, but not IFNγ require active protein kinase C. Moreover, activation of monocytes by IL 2 and not by IFNγ is associated with an increase in CSF1 receptor. Exogenous CSF1 synergized with IL 2 and not IFN-gamma in the activation of cytotoxic human monocytes. We have also shown that the β chain of the IL 2 receptor is constitutively expressed on human monocytes. Overall, these results indicate that the interaction of IL 2 with the β chain is sufficient to trigger the cytolytic activity and the expression of CSF1 receptor, and that CSF1 can further augment the activation of IL 2-activated monocytes. In contrast, IL 2 does not activate mouse macrophages or augments CSF1 receptor expression, although it synergizes with IFNγ in triggering cytolytic activity. These results suggest that in mouse macrophages induction of the α chain of the IL 2 receptor by IFNγ is needed for the response of these cells to IL 2.

Studies have also been performed on the expression of HIV-LTR binding protein during macrophage activation. We found that macrophages constitutively express nuclear proteins binding to HIV-LTR. Moreover, activation of macrophages by endotoxins induces the synthesis of a new protein involved with HIV-LTR binding activity. These results provide the first evidence that the expression of HIV-LTR binding proteins can be modulated during the activation process and suggest that such protein may be important in regulating the expression of HIV in macrophages.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Luigi Varesio	Visiting Scientist	IS, LMI, NCI
Michael Clayton	Microbiologist	IS, LMI, NCI
George Cox	BTP Fellowship	IS, LMI, NCI
Patricia Latham	BTP Fellowship	IS, LMI, NCI
Igor Espinoza	Visiting Fellow	IS, LMI, NCI

OBJECTIVES

The objectives of the research have been: 1) to investigate the molecular events leading to activation/differentiation of human and murine macrophages and macrophage-like cell lines; 2) to analyze the expression HIV-LTR binding proteins in macrophages; and 3) to study the expression and the function of the IL 2 and CSF1 receptor on macrophages.

MAJOR FINDINGS

I. Control of Gene Expression During Macrophage Activation

The expression of a number of genes involved in the response of macrophages to the potent activators of tumoricidal activity LPS and γIFN has been studied. We have used the murine macrophage cell line ANA1, generated in our laboratory by infecting the bone marrow of C57B1/6 mice with the J2 retrovirus that expresses v-raf and v-myc oncogenes. Initially we focused on the regulation of the expression of the c-fms proto-oncogene, that codes for the CSF1 receptor, and on the 2'5' oligo-A synthetase. That is an enzyme implicated in the anti-viral response as well as in the tumoricidal activity of macrophages. We have shown that the expression of c-fms proto-oncogene is down-regulated by LPS. This effect becomes evident with doses of LPS lower than 100 ng/ml and reaches the maximal levels at 1 μg/ml. LPS decreases c-fms mRNA within 6 hours and shows maximal effect at 18 hours. γIFN, although per se ineffective, synergizes with LPS at doses of LPS that are ineffective. We have demonstrated that transcriptional mechanisms control c-fms down-regulation in response to LPS and to LPS plus γIFN. The analysis of c-fms expression in conditions of inhibition of RNA or protein synthesis rules out the involvement of other post-transcriptional events, elicited by LPS, in c-fms regulation. We think this could be a mechanism by which macrophages modulate their own response to stimuli provided by CSF1. The 2'5' oligo-A synthetase is involved in the antiviral effect induced by IFNs. The activation of 2'5' oligo-A synthetase in presence of double stranded RNA stimulates the activity of a specific RNase (RNaseF). Data obtained in our laboratory suggest that, besides the antiviral activity, stimulation of RNaseF upon 2'5' oligo-A synthetase activation be normally involved in the turnover of cellular transcripts. In particular activation by LPS treatment strongly increases the expression of 2'5' oligo-A synthetase at levels higher than those induced by γIFN. LPS induction of 2'5' oligo-A

synthetase appears at 4 hours after treatment and constantly increases up to 24 hours. Although gamma-IFN at lower extent follows the same pattern of induction, when used in combination with LPS, it prevents or strongly reduces LPS induction of 2'5' oligo-A synthetase. We are presently analyzing the molecular mechanisms involved in the 2'5' oligo-A synthetase induction by LPS and γIFN.

IL 1 and TNFα are induced in macrophages by LPS; however, it is unclear whether similar mechanisms control the expression of both genes. We demonstrated the existence of differential regulation of LPS induced IL 1 and TNFα mRNA expression and protein production in murine macrophages based on the use of inhibitors of second messenger pathways. Northern blot analysis was performed with total RNA obtained from murine (C57Bl/6) peritoneal macrophages stimulated in vitro with LPS with or without an inhibitor of protein kinase C (PKc) (1-(5-isoquinolinesulfonyl)-1-methylpipazine hydrochloride; H7) or an inhibitor of calmodulin (CaM)-dependent kinase (N-(6-amino-hexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride; W7). Northerns were analyzed with probes for IL 1α and IL 1^b and TNFα. The expression of the three cytokine mRNA by LPS was inhibited in a dose response manner by H7. In contrast, the expression of IL 1 mRNA, but not TNFα mRNA, was blocked by treatment with W7. Parallel studies monitoring biologic activities of these two cytokines confirm the mRNA data. PKc inhibitors, H7 and retinal, block both IL 1 and TNFα protein production and inhibitors of CaM kinase, W7, N-(6-aminobutyl)-5-chloro-2-napthalenesulfonamide, calmidazolum, and trifluoperazine dichloride inhibit only IL 1 production. These data suggest that both PKc and CaM kinase dependent pathways are involved in the induction of IL 1 mRNA by LPS. In contrast, TNFα expression appears to be PKc dependent but not CaM kinase dependent.

The effects of IFNγ on c-fos mRNA expression in fresh peritoneal macrophages and on the immortalized macrophage cell line ANA-1 were investigated. IFNγ inhibited the augmentation of c-fos mRNA by TPA, LPS or Calcium Ionophore in fresh non-proliferating macrophages. Under the same experimental conditions, γgamma induced oligo-A synthetase mRNA and did not affect the constitutive expression of TGF beta mRNA indicating that IFNγ did not induce general degradation of mRNAs. IFNγ inhibited the augmentation of c-fos mRNA expression by TPA in the ANA-1 macrophage cell line. Run-off experiments with the ANA-1 cell line indicate that c-fos was constitutively transcribed. However, IFNγ did not inhibit the constitutive or TPA-induced c-fos transcription. These results indicated that IFNγ treated macrophages are fully responsive to TPA, and these results indicated that the control of c-fos mRNA augmentation TPA occured on the post-transcriptional level. This IFN effect may represent very important physiological control mechanism of c-fos proto-oncogene expression in macrophages.

II. Expression of Nuclear Proteins Binding HIV-LTR

Electrophoretic mobility shift assays were performed to investigate protein factors that interact with the different portion of HIV-1 LTR. The assay involves the incubation of nuclear extracts with ^{32}P end-labelled specific DNA fragment. Protein DNA complexes are separated by electrophoresis through a low ionic strengthened reducing polyacrylamide gel and visualized by autoradiography. The DNA protein complex migrates more slowly than free DNA. Since

monocytes and macrophages can serve as a reservoir for HIV-1 and a vehicle for the dissemination of the virus, we analyzed different regions of HIV-1 LTR for their ability to bind nuclear proteins from GG2EE murine macrophage cell lines that have been immortalized from mouse bone marrow. DNA shifting gels were established utilizing the 219 bp EcoRV/EcoRV fragment of HIV-1 LTR. This fragment contains region homologous to IL 2, IL 2R and IFN-gamma promoter region, two of the three sequences related to the AP-1 binding sites, as well as the negative regulatory region. Upon incubation of the fragment with the nuclear extract of GG2EE cells, two major bands shifted (A and C) and a minor band (B) can be seen demonstrating the presence of constitutive proteins in GG2EE nuclei that can bind the HIV-1 LTR. Eighteen hours treatment with LPS (10 μg/ml) induces a dramatic increase of the minor band (B) in a dose dependent manner. The fine specificity of the binding of nuclear proteins to HIV-1 LTR was examined by competition experiments. Binding reactions were carried out in the presence of an excess of different nonradioactive competitors. Hundred-fold excess of the 219 bp of unrelated DNAs inhibited the formation of shifted bands. To investigate whether the induction of the proteins binding to the 219 bp fragment requires de novo protein synthesis GG2EE cells were pretreated for 30' with the translation inhibitor cycloheximide and then stimulated with LPS. When GG2EE cells were stimulated with LPS in presence of cycloheximide, the increase in the intensity of band B was completely abrogated suggesting that active protein synthesis is required for this LPS response. These results provide the first evidence that macrophages express multiple proteins recognizing the 219 bp EcoRV fragment of the HIV-LTR and that LPS can modulate their expression. It is possible that LPS may change the expression of the HIV-virus in monocytes and the viral production by HIV-infected monocytes may vary depending upon the activation stage.

The influence of cytokines on the expression of HIV-LTR linked to the marker gene chloramphenicol acetyl transferase (CAT), LTR-CAT, in a transfected human promonocyte cell line, U937 has been studied. Results indicate that the LTR regulatory region of the HIV gene in this monocyte cell line, as monitored by LTR-CAT, can be up-regulated by TNFα and IL 1α. These results provide direct evidence that cytokines can modulate HIV expression in monocytic cells.

III. Expression of Tumoricidal Activity by Human Monocytes

Monocytes are mature phagocytic macrophages which can be stimulated by lymphokines such as IFNγ or IL 2 to express tumoricidal activity and the secretion of cytostatic cytokines such as IL 1, TNF, and TGFβ. The expression of such activated function is affected by the usual physiology of monocyte differentiation to tissue macrophage. Experiments in the past year have focused on:

1. The stimulus-specific response of monocytes leading to the expression of antitumor activity and the modulating effects of substrate.

Results indicate that human monocytes obtained by centrifugal elutriation (precluding the need for adherence) to high purity (>90%) do not express spontaneous cytotoxicity as fresh or in vitro differentiated cells. In both cases, they can be induced to become tumoricidal after stimulation by IFNγ and IL 2. These stimuli are at least additive when administered together.

Adherence can inhibit the expression of tumoricidal activity and factors which influence adherence can reduce tumoricidal activity. These results demonstrate that environmental factors may be as important as activating cytokines in determining the cytolytic activity of monocytes.

2. Human Monocyte Activation and Gene Expression

IFNγ or IL 2 activate human monocytes to a cytotoxic stage. The signal transduction pathway involved in the activation of human monocytes by these cytokines seems to be different. IFNγ can activate human monocytes in the presence of protein kinase C (PK-C) inhibitors, suggesting that IFNγ induced monocyte cytotoxicity, does not require active PK-C. In contrast, the activation of monocytes by IL 2 was partially abrogated by inhibitors of PK-C suggesting that PK-C was necessary for IL 2 induced monocyte cytotoxicity.

Previous studies in our Section have shown the c-fos induction is an early event in murine macrophages. In addition, c-fos induction by PMA is inhibited by PK-C inhibitors. Based upon these data, we investigated whether c-fos expression is related to monocyte activation. Preliminary data suggest that c-fos expression in human monocytes is under control of PK-C, since activators of PK-C induce c-fos mRNA expression after 30' stimulus.

The proto-oncogene c-fms is a marker for cells of the myelomonocytic lineage. We investigated the effects of IL 2 and IFNγ on the expression of c-fms mRNA in human monocytes, a selective modulation of c-fms was found. IL 2 but not IFNγ up-regulate c-fms mRNA expression. This is the first time that a naturally occurring cytokine up-regulates c-fms expression. Since c-fms encodes for CSF1 receptor, we postulated that human monocytes treated with IL 2 could display a different sensitivity to CSF1. We tested this hypothesis and found that CSF1 enhances IL 2 but not IFNγ induced cytotoxicity in human monocytes.

3. Human Monocyte IL 2 Receptor

We investigated the expression of IL 2 receptor (subunit B-chain) on monocytes surface. We found that human monocytes constitutively express B chain (pb). In addition, we found in close agreement with the current literature, that α chain (p55) is inducible after 24 hours in culture in medium alone after γIFN treatment. Preliminary results obtained from cytotoxicity assay using IL 2 as stimulus and TD-27 a monoclonal antibody against the IL 2 receptor B-chain, suggest that such subunit play a major role in the activation of monocytes to a cytolytic stage. In fact TD-27 antibodies could significantly inhibit the activation of monocytes by IL 2. These results demonstrate that the β-chain of IL 2 receptor is present on human monocytes and that is involved in the response of these cells to IL 2.

IV. Expression and Function of Interleukin 2 Receptors on v-myc/v-raf-Immortalized Murine Macrophages

Although it is clear that the monocyte expresses functional IL 2R, it is not known whether the more mature tissue macrophage expresses structurally similar IL 2R, or whether the biological consequences of IL 2 exposure are similar for these two closely related but distinct populations of cells. Therefore, this

project was undertaken to examine the expression and function of IL 2R on macrophages. A model system consisting of v-myc/v-raf-immortalized murine macrophage cell lines as well as normal murine thioglycollate-elicited peritoneal macrophages has been utilized for these studies since this is a well characterized and convenient source of macrophages.

Early studies revealed that the ANA-1 macrophage cell line expresses specific mRNA for the p55 subunit of the IL 2R following a 2-5 hour treatment with 100 U/ml of IFNγ but not medium alone. Additional time-response studies indicated that ANA-1 macrophage exposed to prolonged treatment (18-25 hours) with IFNγ do not express p55 mRNA. Utilizing monoclonal antibodies and flow cytometric analysis and observed that the expression of the p55 subunit of the IL 2R on the surface of immortalized murine macrophage cell lines can either be induced by IFNγ treatment (GG2EE cell line) or be augmented by IFNγ treatment (ANA-1 cell line). Functional studies have indicated that IL 2 can act as a co-stimulus with IFNγ for the induction of the tumoricidal activity of immortalized macrophages. The observation that IL 2 alone does not induce the tumoricidal activity of these macrophage populations indicates at least one difference between the IL 2R system of macrophage versus monocytes. That IL 2 can synergize with IFNγ for the induction of tumoricidal activity in the C3H/HeJ mouse-derived GG2EE cell line or C3H/HeJ-derived peritoneal macrophages indicates that IL 2 can circumvent the genetic defect of the LPS-hyporesponsive C3H/HeJ mouse. Interestingly, while IL 2 synergizes with IFNγ for the induction of the tumoricidal activity of immortalized macrophages it also synergizes with IFNγ for the inhibition of macrophage cell line proliferation. This suggests a possible role for IL 2 as an autocrine factor for the proliferation and differentiation of these cells.

PUBLICATIONS

Varesio L, Blasi E, Gandino L, Bottazzi B, Carbone A, Clayton M, Radzioch D. Oncogenes in the immortalization and activation of macrophages. In: Tonini GP, Massino L, Cornaglia-Ferraris P, eds. Oncogenes in Pediatric Tumors. New York: Harwood Academic, 1988;157-70.

Blasi E, Radzioch D, Varesio L. Inhibition of retroviral mRNA expression in the murine macrophage cell line GG2EE by biological response modifiers. J Immunol, 1988;141:2153-7.

Kovacs EJ, Radzioch D, Young HA, Varesio L. Control of LPS induced interleukin 1 and tumor necrosis factor mRNAs expression by inhibitors of second messenger pathways in murine macrophages. In: Powanda MC, Oppenheim JJ, Kluger MJ, Dinarello C, eds. Monokines and Other Non-Lymphocytic Cytokines. New York: Alan R Liss, 1988;55-60.

Kovacs EJ, Radzioch D, Young HA, Varesio L. Differential inhibition of IL 1 and TNFα mRNA expression by agents which block second messenger pathways in murine macrophages. J Immunol 1988;141:3101-5.

Blasi E, Varesio L, Wiltrout RH. Tumor formation by a murine macrophage cell line immortalized in vivo by v-raf and v-myc oncogenes. Cancer Immunol Immunother 1988;27:109-13.

Kovacs EJ, Beckner SK, Longo DL, Varesio L, Young HA. Cytokine gene expression during the generation of human lymphokine-activated killer cells: Early induction of interleukin 1β by interleukin 2. Cancer Res 1988;49:960-6.

Chang Z, Bonvini E, Varesio L, Holden HT, Herberman RB. Differential in vitro modulation of suppressor and antitumor functions of mouse macrophages by lymphokines and/or endotoxins. Cell Immunol 1988;114:282-92.

Peppoloni S, Blasi E, Ortaldo JR, Rapp UR, Riccardi C, Varesio L. In vitro proliferation of human large granular lymphocytes with v-raf/v-myc recombinant retrovirus. Experientia 1988;44:1013-5.

Varesio L, Bottazzi B, Clayton M, Blasi E, Radzioch D. Molecular events in macrophage cytotoxicity. In: Effector Mechanisms in Tumor Immunity, Torino, Italy, in press.

Ortaldo JR, Young HA, Varesio L. Modulation of $CD3^-$ LGL functions by agonist and antagonists of PKC: Effects on NK and LAK activity and production of IFNγ. J Immunol, in press.

Varesio L, Tonini GP, Blasi E, Bottazzi B, Clayton M, Radzioch D. Advances in macrophage activation. In: Proceedings on New Findings in Research Laboratories. Torini, Italy, in press.

Varesio L, Gonda M, Latham P. Identification and functional characterists of monocytes/macrophages. In: Jones TC eds. ILS 1 Monograph on Pathology Laboratory Animals Hemopoietic System. New York: Springer-Verlag, in press.

Blasi E, Radzioch D, Varesio L. Generation of macrophage cell line from fresh bone marrow cells with myc/raf recombinant retrovirus. Cancer Biochem Biophysics, in press.

Parodi MT, Varesio L, Tonini GP. Morphological changes and cellular differentiation induced by cisplatin in human neuroblastoma cell lines, Cancer Chem Pharmacol, in press.

Wiltrout RH, Varesio L. Activation of macrophages for cytotoxic and suppressive effector functions. In: Oppenheim JJ, Shevach E, eds. Textbook of Immunophysiology: Role of Cells and Cytokines in Immunity and Inflammation. New York: Oxford Press, in press.

Faltynek, CR, Princler GL, Varesio L, Radzioch D. A functional protein kinase C is required for induction of 2'5'A synthetase by recombinant interferon-gamma in Daudi cells. J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09260-07 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Human Inflammatory Cytokines: Production, Properties, and Effects.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. Matsushima	Visiting Scientist	LMI, NCI
Others:	J. J. Oppenheim	Chief	LMI, NCI
	M. Shiroo	Visiting Fellow	LMI, NCI
	A. Samanta	Guest Researcher	LMI, NCI
	C. Zachariae	Guest Researcher	LMI, NCI
	K. Hirose	Guest Researcher	LMI, NCI
	N. Mukaida	Guest Researcher	LMI, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*

NCI, Bethesda, MD (E. Appella); DOD, Ft. Detrick, Frederick, MD (A. O. Anderson); Dainippon Pharmaceutical Co. Ltd., Japan (M. Yamada).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Immunobiology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of the signal transduction through IL 1 receptors and post receptor molecular events have established that IL 1 rapidly phosphorylates 65 and 74 kDa cytosolic proteins at serine residues in human peripheral blood mononuclear cells (PBMC) through activation of an unidentified protein kinases which is distinct from protein kinase C, protein kinase A, or casein kinase. The phospho 65 kDa protein has been purified, molecularly cloned, and identified to be 1-plastin. Tumor necrosis factor which has many over-lapping biological activities with IL 1 also activates the same serine kinase and phosphorylates the identical cytosolic proteins. We have been investigating the basis for the growth inhibitory/ cytocidal effect of IL 1 and TNF on several types of human tumor cell lines. We have further confirmed that the regulation of a rate-limiting enzyme for polyamine synthesis, ornithine decarboxylase and a mitochondria oxygen detoxifying enzyme, manganese superoxide dismutase precedes cell growth inhibition by IL 1 and TNF; and the regulation of these enzymes by IL 1 and TNF is very specific. The identification of novel IL 1/TNF inducible cytokines has enabled us to purify and molecularly clone the cDNAs of two novel leukocyte chemotactic and activating factors, i.e. neutrophil/lymphocyte chemotactic and activation factor (newly named interleukin 8), and monocyte chemotactic and activating factor (termed MCAF). Biological, biochemical and molecular biological properties of these molecules have been performed and the receptors for these molecules have been partially characterized.

PROJECT DESCRIPTION

PERSONNEL

Kouji Matsushima	Visiting Scientist	IS, LMI, NCI
Joost J. Oppenheim	Chief	OC, LMI, NCI
Masahiro Shiroo	Visiting Fellow	IS, LMI, NCI
Ajoy Samanta	Guest Researcher	IS, LMI, NCI
Claus Zachariae	Guest Researcher	IS, LMI, NCI
Kunitaka Hirose	Guest Researcher	IS, LMI, NCI
Naofumi Mukaida	Guest Researcher	IS, LMI, NCI
Lorraine Thompson	Guest Researcher	IS, LMI, NCI
Geeta Chaudri	Special Volunteer	IS, LMI, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI

OBJECTIVES

We are attempting to gain a better understanding of how signals are transduced by IL 1 receptors and result in altered gene expression, cell function and growth, and to establish the biological and molecular nature of IL 1/TNF inducible newly defined cytokines, IL 8 and MCAF.

MAJOR FINDINGS

1. Signal transduction through IL 1 receptor and post-receptor molecular events.

a. Identification of protein phosphorylation induction by IL 1.

We previously reported the identity of the receptor for IL 1a and IL 1b, the down regulation of IL 1 receptor by IL 1 itself, and the upregulation of IL 1 receptor by glucocorticoids and prostaglandins E. We have found that recombinant human IL 1α at as low a concentration as 1 ng/ml specifically and induces predominantly the phosophorylation of 65 and 74 kDa cytosolic proteins in glucocorticoid pretreated human PBMC. The phosphorylation occurs within 1 min and reached a maximum at 10 min after the stimulation of PBMC with IL 1α. Thin layer chromatography of hydrolysate of 32P-labeled 65 and 74 kDa proteins showed that only serine residues are phosphorylated. The cytosolic 65 kDa phosphoprotein has been purified, molecularly cloned and identified to be 1-plastin.

b. IL 1 and TNF activate an identical serine kinase which is distinct from protein kinase A or C.

It has been observed that two identical cytosolic proteins are phosphorylated in human PBMC after stimulation with TNF or phorbol esters, but not with diacylglycerol, prostaglandins E, or forskolin. The induction of protein phosphorylation by IL 1 was not blocked by a protein kinase C inhibitor, H-7. There was no increase in cAMP levels after the stimulation of cells with

IL 1 or TNF. These results suggest that the phosphorylation of 65 and 74 kDa proteins following stimulation by IL 1 and TNF is not mediated through protein kinase A or protein kinase C activation. This hypothesis has been further supported by directly identifying a distinct protein kinase which is activated in human PBMC by stimulating with IL 1, TNF, or phorbol esters, and is able to phosphorylate 65 kDa protein in vitro. This partially purified protein kinase (p65 kinase) is located in the cytosol and distinct from protein kinase A, C or casein kinase and appears to participate in signal transduction by IL 1 and TNF.

2. Molecular analysis of the growth inhibition of tumor cells by IL 1 and TNF.

We previously reported that IL 1 exerts directly an antiproliferative effect on several types of human tumor cells in vitro. We found that the suppressive effect of IL 1 on ornithine decarboxylase (ODC) activity anteceded and correlated well with its effect on DNA synthesis of tumor cells. Furthermore, putrescine, a product of the ODC reaction and a precursor of polyamines, was able to overcome the anti-proliferative action of IL 1 on some tumor cell lines, such as A375 human melanoma cells. We also found that mitochondrial associated enzyme, manganese superoxide dismutase (MnSOD) is a prominent early inducible protein in A375 cells after the treatment with IL 1. Since no other oxygen detoxyfying enzymes, mitochondrial associated proteins or DNA repair enzymes are induced by IL 1, including Cu/Zn SOD, extracellular SOD, catalase, gluthathione peroxidase, glutathione-S-transferase, heat shock proteins, poly ADP-ribose synthetase, cytochrome b, NADH dehydrogenase, DNA polymerase b, and excision repair gene ERCC-1, the induction of MnSOD by IL 1 is very specific. We speculate that overproduction of hydrogen peroxide by MnSOD in mitochondria may generate OH peroxide which is not readily converted to water by catalase or gluthathione peroxidase. The protective activity of putrescine may be due to chelating activity for iron ion which is essential to the conversion of hydrogen peroxide to OH radicals. TNF which has a similar biological effect on tumor cells with IL 1 also down-regulates ODC activity and induces MnSOD in various types of cells.

3. Purification and molecular cloning of two novel IL 1/TNF inducible leukocyte chemotactic and activating factors.

Recruitment of leukocytes into tissues is dependent on the local release of leukocyte activating and chemotactic mediators in response to injury-induced or immunologically mediated inflammatory reactions. Although both IL 1 and TNF were reported to be directly chemotactic for human neutrophils, monocytes, and T lymphocytes and possibly identical to major chemotactic factors in human leukocyte conditioned media, we have recently purified neutrophil, monocyte, and lymphocyte chemotactic factors to homogeneity from the conditioned media of human PBMC or monocytic cell line stimulated with mitogens and have shown that these chemotactic factors are structurely distinct from any known cytokines, including IL 1 or TNF. Neutrophil chemotactic factor was structurally identical to T lymphocyte chemotactic factor and has been renamed IL 8. cDNA cloning of IL 8 has been performed and the cDNA encodes a 99 amino acid precursor form of IL 8. There is a typical signal peptide at the amino terminal end and the mature processed soluble extracellular form of IL 8 consists of 72 amino acids. IL 8 shows high amino acid sequence homology to several human factors, such as b-thromboglobulin, platelet factor 4, IP 10 and Gro/MGSA. Biologically active

IL 8 has been chemically synthesized, and expressed in E. coli, and CHO cells. The chromosomal IL 8 gene has been also cloned and mapped to chromosome #4, q 12-21. Northern blotting analysis revealed that IL 8 mRNA is inducible within 30 min and reached maximal at 1 to 2 hours in human PBMC, dermal fibroblasts, keratinocytes, and endothelial cells in response to IL 1 or TNF. Biologically active IL 8 was also detected in the conditioned media from IL 1 or TNF stimulated cells. When recombinant IL 8 was injected subcutaneously, margination and emigration of neutrophils and lymphocytes from postcapillary venules was observed at the injected site. Low doses (10 ul of 1 ng/ml/site) predominantly induced lymphocyte emigration, whereas higher doses (10 ul of 100 ng/ml/site) predominantly induced neutrophil emigration. In addition, accelerated accumulation of small lymphocytes into high endothelial venules of regional draining lymph nodes was observed after subcutaneous injection of IL 8. IL 8 also causes lysosomal enzyme release from neutrophils in the presence of cytochalasin B, increases Candida Albicans killing by neutrophils, upregulates Mac-1 expression on neutrophils in vitro and induces rapid plasma leakage in vivo. Receptors for IL 8 on human peripheral blood neutrophils which are distinct from the receptors for IL 1, TNF, C5a, LTB4, PAF, and fMLP have been identified. Neutrophils express 20,000/cell with a Kd of 8×10^{-10}M. Lower number of IL 8 receptors (<500/cell) have been also detected on human peripheral blood T lymphocytes, immature myeloid cell lines, and an Epstein Barr virus transformed B lymphocyte cell line.

A monocyte chemotactic factor has also been purified and molecularly cloned, and recombinant molecules have been expressed in CHO cells. Since this novel monocyte chemotactic factor also induces superoxide production, lysosomal enzyme release, and increases the cytostatic activity of monocytes against several types of human tumor cells in vitro, we termed this factor monocyte chemotactic and activating factor (MCAF). MCAF mRNA encodes a 99 amino acid precursor MCAF. There is a typical signal peptide at the amino terminal and the mature form of MCAF consists of 76 amino acids. MCAF shows high amino acid sequence homology with several other new cytokines, such as JE, LD 78, RANTES, TCA-3 and ACT-2. MCAF also shows significant amino acid sequence homology with IL 8 (21%). MCAF mRNA is also rapidly induced by stimulating human PBMC, dermal fibroblasts, and endothelial cells with IL 1 or TNF. When natural pure human MCAF was injected subcutaneously into rats, selective emigration of monocytes was observed starting at 6 hrs.

At lower doses IL 1/TNF cause the migration of mixture of lymphocytes and neutrophils, and higher doses of IL 1/TNF caused predominantly neutrophil infiltration by 2 to 6 hrs in a dose dependent manner. Since IL 1 and TNF lack direct leukocyte chemotactic activity in vitro, the in vivo cell migration activity of IL 1 and TNF may be mediated by the induction of IL 8 and MCAF by IL 1/TNF.

SIGNIFICANCE

1. Study of the identification of in vivo substrates of protein kinase which is activated by stimulating human PBMC with IL 1 has shown that IL 1, TNF and PMA phosphorylate common 65 and 74 kDa cytosolic proteins. This 65 kDa protein has been identified to be l-plastin which has been reported to be widely expressed in leukocytes and transformed fibroblasts. Although the receptors for IL 1 and

TNF are distinct, our study has shown that IL 1 and TNF activate a common cytosolic serine kinase to phosphorylate the 65 kDa protein. This may explain why IL 1 and TNF have many overlapping biological activities. The identified IL 1/TNF activated serine protein kinase is at least distinct from protein kinase C, protein kinase A, or casein kinase.

2. Molecular analysis of the growth inhibition of tumor cells by IL 1 and TNF has shown that IL 1 and TNF specifically and rapidly regulate two cell associated enzyme activities, i.e. ODC and MnSOD activities, prior to cell growth inhibition. Decrease of polyamine levels caused by down-regulation of ODC activity by IL 1 lead directly to cell growth inhibition and also accelerate the generation of cell-toxic OH radicals from hydrogen peroxide which is generated by induced MnSOD.

3. Identification of novel IL 1/TNF inducible cytokines has led us to purify and molecularly clone two novel leukocyte chemotactic and activating cytokines, i.e. IL 8 and MCAF. These studies have revealed the cell sources, some of the functions and molecular structures of leukocyte derived leukocyte chemotactic factors which have been described since 1972. These new cytokines may be useful and antitumor agents and/or anti-HIV agents. Since these cytokines have very strong proinflammatory activities, the development of antagonists to these molecules may also prove important to the treatment of some inflammatory diseases.

PROPOSED COURSES

It is the overall aim of this laboratory to investigate the mechanism by which human inflammatory cytokines such as IL 1, TNF, and IL 1/TNF inducible cytokines contribute to host defense and tumor immunity. We are studying the mechanism of signal transduction of IL 1 and TNF receptors, post-molecular intracellular events, the regulation of IL 1/TNF inducible genes, and the mechanism of tumor cell growth inhibition by IL 1 and TNF. We are going to pursue the following studies:

1. Study of signal transduction mechanism mediated by IL 1/TNF receptors and post receptor molecular events.

a. Purification of IL 1/TNF inducible cytosolic protein kinase which phosphorylates 65 kDa protein (1-plastin).

b. Establishment of the association of the identified IL 1/TNF inducible protein kinase with IL 1/TNF receptors.

c. Establishment of the function of 5'-flanking region of IL 8 chromosomal gene, identifying IL 1/TNF inducible nuclear binding factors, and IL 1/TNF response element(s) on IL 8 gene.

d. Examining the possible modification of IL 1/TNF inducible nuclear factors by the above 65 kDa protein kinase.

2. Molecular analysis of tumor growth inhibition by IL 1/TNF.

a. Examining possible mitochondrial DNA fragmentation after the treatment of tumor cells with IL 1 or TNF.

b. Transfection of sense and anti-sense DNA or MnSOD to several types of cells and examining the change in sensitivity of these cells to IL 1, TNF, irradiation, and chemotherapeutic agents.

c. Genomic sequence of MnSOD and molecular analysis of the induction of MnSOD by IL 1 and TNF.

3. IL 1 and TNF inducible leukocyte chemotactic and activating factors, IL 8 and MCAF.

a. Biological activities in vitro and in vivo, particularly antitumor and anti-infectious activities.

b. Study of the production of these factors in various human inflammatory diseases, including psoriasis, rheumatoid arthritis, and uveitis.

c. Purification of IL 8 and MCAF receptors, cDNA cloning and study of the signal transduction mechanism mediated by these receptors.

d. Secondary and tertiary structural analysis of these chemotactic cytokines, modification/truncation and development of antagonistic or agonistic polypeptides.

e. Establishment of the biological activities of related molecules and identification of other IL 1/TNF inducible cytokines.

PUBLICATIONS

Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, Appella E, Kung HF, Leonard EJ, Oppenheim JJ. Molecular cloning of ahuman monocyte derived neutrophil chemotactic factor (MDNFC) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. J Exp Med 1988;167:1882-93.

Modi WS, Masuda A, Yamada M, Oppenheim JJ, Matsushima K, O'Brien SJ. Chromosomal localization of the human interleukin 1α (IL 1α) gene. Genomics 1988;1:310-4.

Merrill JE, Matsushima K. Autocrine regulation of a cloned human oligodendroglioma cell line by interleukin 1. J Biol Regulators Homeostatic Agents 1988;2:77-86.

Endo Y, Matsushima K, Onozaki K, Oppenheim JJ. Role of ornithine decarboxylase in the regulation of cell growth by interleukin 1 and tumor necrosis factor. J Immunol 1988;141:2342-8.

Akahoshi T, Oppenheim JJ, Matsushima K. Interleukin 1 (IL 1) stimulates its own receptor expression on human fibroblasts through the endogenous production of prostaglandin(s). J Clin Invest 1988;82:1219-24.

Masuda A, Longo DL, Kobayashi Y, Appella E, Oppenheim JJ, Matsushima K. Induction of mitochondrial manganese superoxide dismutase by interleukin 1. FASEB J 1988;2:3087-91.

Tanaka S, Robinson EA, Yoshimura T, Matsushima K, Leonard EJ, Appella E. Chemical synthesis and biological characterization of monocyte-derived neutrophil chemotactic factor (MDNCF). FEBS Ltr 1988;236:467-70.

Matsushima K, Shiroo M, Lew W, Kobayashi Y, Akahoshi T, Oppenheim JJ. Detection of protein phosphorylation in human peripheral blood mononuclear cells in response to interleukin 1 (IL 1). In: Powanda MC, Oppenheim JJ, Kluger MJ, Dinarello CA, eds. Progress in Leukocyte Biology, 1988;8:229-34.

Yoshimura T, Robinson EA, Appella E, Matsushima K, Showalter SD, Skeel A, Leonard E. Three forms of monocyte-derived neutrophil chemotactic factor (MDNCF) distinguished by different lengths of the amino terminal sequence. Mol Immunol 1989;26:87-93.

Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K. Identity of chemotactic cytokine for T-lymphocytes with neutrophil activating protein (NAP-1): A candidate interleukin-8. Science 1989;243:1464-6.

Samanta AK, Oppenheim JJ, Matsushima K. Identification and characterization of a specific receptor for monocyte-derived neutrophil chemotactic factor (MDNCF) on human neutrophils. J Exp Med 1989;169:1185-9.

Furutani Y, Nomura H, Notake M, Oyamada Y, Fukui T, Yamada M, Larsen CG, Oppenheim JJ, Matsushima K. Cloning and sequenceing of the cDNA for human monocyte chemotactic and activating factor (MCAF). Biochem Biophys Res Commun 1989;159:249-55.

Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. J Exp Med 1989;169:1485-90.

Larsen CG, Zachariae COC, Oppenheim JJ, Matsushima K. Production of monocyte chemotactic and activating factor (MCAF) by human dermal fibroblasts in response to interleukin 1 or tumor necrosis factor. Biochem Biophys Res Commun 1989;160:1403-8.

Matsushima K, Larsen CG, Samanta AJ, Mukaida N, Anderson AO, Oppenheim JJ. An interleukin 1/tumor necrosis factor inducible inflammatory cytokine, interleukin 8. In: TNF and Related Cytokines. Switzerland: Krager Publications, in press.

Matsushima K. A novel interleukin 1/tumor necrosis factor inducible chemotactic cytokine, interleukin 8. Med Immunol, in press.

Matsushima K. Interleukin 1 receptor: Regulation of the expression, structure and signal transduction. Med Immunol, in press.

Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor (MDNCF), interleukin 8. J Immunol, in press.

Furuta R, Yamagishi J, Kotani J, Sakamoto F, Fukui T, Matsui Y, Sohmura Y, Yamada M, Yoshimura T, Larsen CG, Oppenheim JJ, Matsushima K. Production and characterization of recombinant human neutrophil chemotactic factor. J Biochem, in press.

Larsen CG, Anderson AO, Oppenheim JJ, Matsushima K. Production of neutrophil activating protein-1/interleukin 8 by human dermal fibroblasts and keratinocytes in response to interleukin 1 or tumor necrosis factor. Immunology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09287-05 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytokines and the Immune Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. K. Durum	Senior Staff Fellow	LMI, NCI
Other:	L. Takacs	Visiting Fellow	LMI, NCI
	L. Bristol	IRTA Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (F. Aiello, W. Gotlieb, K. Muegge, M. Smith); University of Pennsylvania (T. Williams, J. Kant); UCSD (M. Karin); LEI, BRMP, DCT, NCI (H. Young); NIAID (U. Siebenlist).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Immunobiology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interleukin 1 (IL 1) induces a variety of responses in different cell types. An important action of IL 1 in the immune response is the induction of interleukin 2 (IL 2) production by T lymphocytes; here IL 1 acts as a costimulus together with a signal from the antigen receptor. To understand this costimulation by IL 1, we have analyzed how IL 1 activates the promoter on the IL 2 gene. We showed that IL 1 induced increases in message for *jun*, whereas the antigen receptor induced *fos*. The *jun* and *fos* proteins interact, forming the AP-1 transcription factor, which then activates the promoter on the IL 2 gene. This explains why two signals, IL 1 and antigen, are required for T cells to produce IL 2. Induction via AP-1 may be a general mechanism of IL 1 action, since we have observed this *jun* induction in two other cell types, a glioblastoma that responds to IL 1 by vigorously increasing growth, and a hepatoma cell line that responds to IL 1 by synthesizing acute phase proteins.

To further our understanding of how IL 1 acts on T cells, we have cloned IL 1-induced genes from T cells using subtractive hybridization. A μ gt11 library of several hundred such clones is currently being analyzed. This may lead to identifying new cytokines, receptors, nuclear factors, and regulators of cell growth and differentiation.

We previously found IL 1 message in the thymus. We have now asked what role IL 1 plays in T cell development in this organ. We show that, in vitro, IL 1 acts on immature thymocytes ("double-negative" cells), inducing their proliferation and differentiation to mature helper T cells ($CD4^+$, α/β). This suggests a possible application for IL 1 in increasing numbers of helper T cells in Acquired Immune Deficiency Syndrome.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Scott K. Durum	Senior Staff Fellow	IS, LMI, NCI
Laszlo Takacs	Visiting Fellow	IS, LMI, NCI
Lynn Bristol	IRTA Fellow	IS, LMI, NCI

OBJECTIVES

1. How does IL 1 control the promoter on the IL 2 gene?

2. What novel genes are induced by IL 1?

3. What is the role of IL 1 in T cell development in the thymus?

METHODS EMPLOYED

1. LBRM, a mouse T cell line, was transfected with various constructs (the IL 2 promoter, various deletion mutants or the AP-1 motif) linked to the reporter gene chloramphenicol acetyltransferase (CAT). Cells were stimulated with IL 1 and PHA (to cross-link antigen receptors). CAT enzyme activity was then analyzed as a measure of promoter activity. To identify DNA-binding proteins, band shift assays were performed using various oligonucleotides and nuclear protein preparations. RNA blot analysis was performed to identify messenger RNAs.

2. Mouse T cells were stimulated for 4 hr with IL 1 and cycloheximide. cDNAs were produced, then non-IL 1-induced sequences were subtracted using mRNA from T cells given cycloheximide alone, and separation on hydroxyapatite. A library was prepared in λ gt11.

3. Immature rat thymocytes ("double-negative" cells) were prepared by density separation and antibody treatments. Double-negative cells were placed in culture with a variety of stimulants and cytokines. Proliferation was measured by radiothymidine incorporation. Differentiation was assessed by antibody staining and FACS analysis. T cell receptor (TcR) expression was analyzed by RNA blot analysis with probes for TcR α, β, γ and δ.

MAJOR FINDINGS

1. We have shown that IL 1 induced increases in the message for jun, whereas the antigen induced fos. The jun and fos proteins interact, forming the AP-1 transcription factor, which then activates the promoter on the IL 2 gene. Other cell types also produced jun in response to IL 1; these include a glioblastoma and a hepatoma.

2. We have produced several hundred IL 1-induced cDNAs from T cells. These will be sequenced to determine whether they represent novel genes.

3. We showed that IL 1 has activities on immature thymocytes, inducing proliferation and differentiation to mature helper T cells.

SIGNIFICANCE

1. IL 1 has many important activities on different cell types in the body. However, the intracellular mechanism of IL 1 action on cells has not been understood, despite considerable effort from a number of laboratories studying the conventional signal transduction pathways. Our study is the first to indicate how IL 1 activates a gene via nuclear factors, and concludes that induction of jun is a critical event in IL 1 action. Since AP-1 sites are present in the regulatory regions of many genes, this suggests how IL 1 could have so many actions. It is also the first demonstration that the two components of AP-1, jun and fos, can be controlled independently. These studies will lead to further experiments to understand the early events in IL 1 action: what is the intermediate pathway leading from the IL 1 receptor on the membrane to the increase in jun message?

2. We have cloned a large number of IL 1 induced cDNAs from T cells. Once a cDNA is sequenced, we will know whether it is novel. This approach will greatly enhance our understanding of the action of IL 1 on cells and has the potential to identify novel cytokines, receptors, nuclear factors and regulators of cell growth and differentiation.

3. The development of T cells in the thymus is a poorly understood process of differentiation, selection and proliferation. How does the thymus control the number of T cells it produces? We have studied the role of cytokines in this process, and these findings may be useful in AIDS therapy to accelerate the production of $CD4^+$ α/β T cells destroyed by the virus. Thus, IL 1 promoted the production of such cells in vitro, whereas IL 2 induced production of $CD8^+$ γ/δ cells, which may be deleterious.

PROPOSED COURSE

1. We will examine the mechanism of IL 1 induction of jun mRNA. The two main possibilities are that IL 1: a) acts on the jun promoter or b) stabilizes jun mRNA. To study a), we will transfect cells with constructs consisting of the jun promoter linked to CAT. If this construct responds to IL 1, we will use deletion mutants and band shift assays to identify the IL 1-responsive element. If this is a novel element, we will characterize the IL 1-induced nuclear factor. If, on the other hand, IL 1 does not act on the jun promoter, we will examine the second possibility: b) since jun mRNA is known to be unstable, we will examine whether IL 1 stabilizes jun mRNA. If this is true, we will study this mechanism by examining proteins that bind to regions of jun mRNA.

Other studies of the IL 1-jun connection will be performed. We will attempt to replace IL 1 signals to cells by transfecting them with a jun expression vector. We will also examine aspects of the pathway leading from the IL 1 receptor to

the active AP-1 transcriptional factor; for example inhibitors of c and a-kinases will be used to test whether these enzymes are involved.

2. Each clone from the IL 1-induced subtraction library will be first tested to determine if its mRNA is detectable in IL 1-stimulated T cells, but absent in unstimulated T cells. Clones that meet these criteria will be partially sequenced until a few "interesting" clones are discovered. Properties of interest would first be novelty, then we would examine a clone for the features of a cytokine, receptor, nuclear factor, kinase or other characteristics revealed by homology to products of known genes. Once a few such gene products are identified, we would focus our efforts on determining their function, and whether they are important in the mechanism of action of IL 1 on a cell.

3. We will apply our knowledge of in vitro cytokine effects to in vivo treatments, with the objective of identifying regimen to accelerate the production of $CD4^+$ cells. Mice will be depleted of T cells, then treated with cytokines. Several depletion methods will be used including: a) treatment with anti-CD4 antibody and b) whole body irradiation. Mice will then be injected with various cytokines, including IL 1, IL 2, IL 3, IL 4, IL 6, IL 7, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon γ (each of these cytokines has been implicated in thymopoiesis by our laboratory or others). At various time points, the recovery of $CD4^+$ cells will be monitored by antibody staining and FACS analysis.

PUBLICATIONS

Ozaki S, Durum SK, Muegge K, York-Jolley J, Berzofsky JA. Production of T-T hybrids from T cell clones: Direct comparison between cloned T cells and T hybridoma cells derived from them. J Immunol, 1988;141:71-8.

Brody DT, Durum SK. A plasma anchoring mechanism for IL 1. In: Powanda M, Oppenheim JJ, Kluger MN, Dinarello CA, eds. Monokines and Other Non-Lymphocytic Cytokines. New York: Alan R Liss 1988;101-7.

Takacs L, Kovacs EJ, Smith MR, Young HA, Durum SK. Detection of IL 1 gene expression by in situ hybridization histochemistry: Tissue localization of IL 1 mRNAs in the normal C57BL/6 mouse. In: Powanda M, Oppenheim JJ, Kluger MJ, Dinarello CA, eds. Monokines and Other Non-Lymphocytic Cytokines. New York: Alan R Liss 1988;89-93.

Tartakovsky B, Finnegan A, Muegge K, Brody DT, Kovacs EJ, Smith MR, Berzofsky JA, Young HA, Durum SK. IL 1 is an autocrine growth factor for T cell clones. J Immunol 1988;141:3863-7.

Harel-Bellan A, Durum SK, Muegge K, Abbas A, Farrar WL. Specific inhibition of lymphokine biosynthesis and autocrine growth using antisense oligonucleotides in TH1 and TH2 helper T cell clones. J Exp Med 1988;148:2309-18.

Takacs L, Kovacs EJ, Smith MR, Young HA, Tartakovsky B, Durum SK. Direct visualization of IL 1 mRNA in tissue sections by in situ hybridization. J Immunol 1988;141:3081-95.

Romero R, Durum S, Duff GW, Mitchell MD. A monokine stimulates prostaglandin E-2 production by human amnion. Postaglandins Leukotrienes Essential Fatty Acids 1988; 34:141-5.

Romero R, Wu YK, Brody D, Oyarzun E, Duff GW, Durum SK. Human deciduum: A source of interleukin 1. Obstetr and Gynecol 1989;73:31-4.

Durum SK, Oppenheim JJ, Neta R. Immunophysiological role of interleukin 1. In: Oppenheim JJ, Shevach EM, eds. Immunophysiology. New York: Oxford Univ Press, in press.

Durum SK, Oppenheim JJ. Macrophage-derived mediators: Interleukin 1, tumor necrosis factor, interleukin 6, interferon and related cytokines. In: Paul W, ed. Fundamental Immunology, in press.

Durum SK, Muegge K, Aiello F, Takacs L. The relationship between T cells and interleukin 1. In: Faist E, ed. Proceedings of the 1st International Congress on the Immune Consequences of Trauma, Shock and Sepsis. W. Germany, Munich, Springer, in press.

Smith MR, Young HA, Kung H-F, Durum SK. Intracellular activities of microinjected cytokines and lymphokines. In: Kaplan JG, ed. The Cellular Basis of Immune Modulation. New York: Alan R. Liss, in press.

Apte R, Oppenheim JJ, Durum SK. Beta endorphin regulates interleukin 1 production and release by murine bone marrow macrophages. Intl J Immunol, in press.

Muegge K, Durum SK. The role of IL 1 in the immune response. In: Bomford R, Henderson B, eds. Interleukin 1, Inflammation and Disease. Amsterdam: Elsevier Press, in press.

Romero R, Brody DT, Oyarzun E, Mazor M, Wu YK, Durum SK. Interleukin 1: A signal for the onset of labor. Am J Obstet Gynecol, in press.

Romero R, Durum S, Dinarello C, Oyarzun E, Hobbins JC, Mitchell MD. Interleukin 1 stimulates prostaglandin biosynthesis by human amnion. Prostaglandins, in press.

Muegge K, Durum SK. Assays for soluble end membrane-bound interleukin 1. In: Coligan JE, Kruisbeck AM, Marqulies DH, Shevach EM, Strober W, eds. Current Protocols in Immunology. New York: Greene Assoc, Inc., in press.

Brody DT, Durum SK. Membrane interleukin 1 (IL 1): IL 1 alpha precursor binds to the plasma membrane via a lectin-like interaction. J Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09251-07 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interactions of Human Retroviruses with Hematopoietic and Adherent Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. W. Ruscetti	Senior Investigator	LMI, NCI
Others:	G. K. Sing	Visiting Fellow	LMI, NCI
	N. C. Lohrey	Microbiologist	LMI, NCI

COOPERATING UNITS *(if any)*

OAD, BRMP, DCT, NCI (D. Longo); LVC, DCE, NCI, (D. Derse); (Upstate Medical Center, Syracuse, NY (B. Poiesz); BCDP, PRI, NCI-FCRF (J. Mikovits, R. Raziuddin); LCMS, PRI, NCI-FCRF (M. Gonda); LBP, NCI (H-F. Kung).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Lymphokine Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.75	.75	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human retroviruses, human imunnodeficiency virus (HIV-I) and human tumor leukemia virus (HTLV-I), are involved in the pathogenesis of chronic, fatal human diseases. We have attempted to better understand the effects of host cell and other viruses on viral replication as well as the interaction between infected cells and the immune system. We have developed in vitro models of viral latency for both HTLV-I and HIV-I. In HTLV-I infected B cells from acute T cell leukemia (ATL) patients, no viral mRNAs are being transcribed, yet intergrated provirus is functional and can be activated to transform other T and B cells. When the virus infects T cells, the virus is expressed; when it infects B cells, it is not expressed. Also, the macrophage, a reservoir of HIV-I infection in acquired immunodeficiency syndrome (AIDS) patients, was shown to be able to restrict HIV-I expression. THP-1, a macrophage cell line, was permissive for HIV-I expression. However, these infected cells become naturally non-productive for HIV 4-6 weeks after infection. Two classes of restricted HIV-I expression were seen: 1) low-level which can be regulated by factors in the nuclei of infected cells and 2) no expression which may be due to methylation of the LTR. Both viruses make proteins which act in *trans* to positively regulate viral transcription. In these latently infected cultures, transactivation of viral transcription is inhibited. This indicates that the host factors play a role in this transcription and these cells either are deficient in positive regulators or possess negative regulators of viral transcription. Understanding the mechanisms of action of negative regulators of viral expression can be useful in developing anti-viral therapies.

PROJECT DESCRIPTION

PERSONNEL

Francis W. Ruscetti	Senior Investigator	LS, LMI, NCI
Garwin K. Sing	Visiting Fellow	LS, LMI, NCI
Nancy C. Lohrey	Microbiologist	LS, LMI, NCI

MAJOR FINDINGS

I. Latent Expression of Functional HTLV-I Provirus in B Lymphocytes.

The HTLV-I and HTLV-II, contain regulatory genes termed tax and rex, whose products function to regulate viral gene expression. The p40tax activates viral transcription directed by the viral long-terminal repeat (LTR) in trans (transactivation) while the p27rex favors production of viral structural over regulatory proteins. HTLV-I has been shown to infect B as well as T cells in vivo. The transactivating and replicative properties of HTLV-I present in HS-1, an infected B-cell line developed from a patient with HTLV-I associated adult T-cell leukemia, were examined. HS-1 showed a lack of transactivation and viral replication, as indicated by the absence of viral RNA and proteins including p40tax. Transformation of fresh cord blood and tonsillar T and B cells by co-cultivation with the lethally irradiated HS-1 cell line showed that the viral genome was not replication defective. Studies on HS-1 viral infection of T and B cells from the same donor showed that in T-cell lines normal transactivation and viral production occurred, although B-cell lines could be used to transform other cells, trans-activation, viral RNA and proteins were absence. Transient co-transfection assays, using an exogenous p40tax expression vector and an HTLV-I LTR-CAT vector revealed a depressed transactivation in B-cell lines containing the HS-1 viral genome compared to B cell lines. The block to transcriptional activation of the virus was removed after treatment of latently infected B-cells with phorbol esters or gamma radiation. Thus, some B cells containing functional provirus appear to be non-permissive for HTLV-I viral transcription. These data show that HTLV-I gene expression can be latent in cells and suggest that host factors play a role in the maintenance of this latent state.

II. Restricted and Latent Expression of HIV-I in Human Monocytes Occur by Different Mechanisms

In monocytes infected with HIV-I and HIV-II, we show that viral expression can occur in several ways. The human monocytoid leukemic cell line, THP-1, which can be differentiated into anchorage dependent, non-dividing macrophages possessing several immune functions, was infected with several isolates of HIV. In all cases, after 10-17 days, THP-1 cultures were producing HIV. Surprisingly, 45-60 days post infection, differences in viral expression were observed among infected cultures: 1) latency (provirus with no viral) expression; 2) restricted expression (intracytoplasmic viral antigens, RNA and virions but no detectable infectious virus released); and 3) continuous

production. In restrictedly infected cells, LTR directed in vitro transcription showed that nuclei contained substances that negatively regulate viral transcription. In addition, viral particles were seen budding into and accumulating within intracytoplasmic vacuoles with little or no extracellular virus suggesting multiple levels of regulation. These restricted cultures had no viral antigens on the cell surface and were not lysed by IL 2 activated large granular lymphocytes while the productively infected cells were efficiently lysed. These cells which released no infectious virus could cause viral-mediated T-cell cytolysis in cell-cell assays suggesting that restricted expression allows persistently infected monocytes to escape immune surveillence and still be capable of evoking T-cell pathology. Latently infected cells made infectious virus after 5-azacytidine exposure, but could not negatively regulate viral transcription in producing cells. Lipopolysaccharide (LPS) could increase viral production in restricted cells but could not activate latently infected cells to produce virus. Thus, both restricted and latent states of HIV expression exist in monocytes and probably occur by different mechanisms.

III. Cytotoxic Effector Mechanisms are Active Against Cells Productively Infected by HIV-I and HTLV-I but not against Restrictedly Infected Cells

AIDS is a viral HIV-I mediated progressive depletion of a helper/inducer T4+ T-cell subset, while acute T-cell leukemia is associated with a viral HTLV-I mediated growth of the same T-cell subset. Since large granular lymphocytes (LGL) with natural killer (NK) activity have been shown to spontaneously lyse several virus infected target cells, the ability of NK cells to lyse both HTLV-I and HTLV-III/LAV infected cells and LGL), with or without pretreatment with recombinant interleukin 2 (IL 2), as well as monocytes, with or without pretreatment with gamma-interferon were employed as effector cells. Both IL 2-activated T cells and NK cells were cytolytic for HTLV-I infected targets. However, only unstimulated LGL showed significant spontaneous activity against HTLV-I infected targets. Only T and B cells and not monocytes were susceptible to infection by HTLV-I. Experiments using cell co-cultures showed that LGL afforded T-cells protection from infection by HTLV-I (as indicated by lack of transformation and viral protein expression).

Similarly, LGL showed spontaneous cytolytic activity against HIV-I infected fresh and cultured targets. This cytotoxicity was considerably augmented by IL 2 treatment of LGL. In contrast, T cells and monocytes were unable to lyse HIV-I targets and only minimal activity was induced by activation. LGL cells, B cells, and monocytes could be infected in vitro by high titers of HIV-I. However, levels of extracellular reverse transcriptase and p24 found in these infected cultures were significantly lower than the levels in T cell cultures. In comparing the productively infected THP-1 (monocytes) with those cells have latent and restricted HIV-I expression, we found IL 2 stimulated LGL killed the HIV-I producing THP-1 but the restricted and latent cultures were not lysed. Collectively, these results indicate that NK cells may play a role in protecting cells against human retroviruses and that viruses with restricted expression can escape immune surveillance.

IV. Characterization of p17 Protein on Plasma Membrane of HIV-Infected Cells by Monoclonal Antibodies and Epitope Mapping

Previous data suggested that neutralizing antibodies to the p17 core protein could be found in AIDS patients and that anti-p17 antibodies could neutralize HIV-infectivity in vitro. More importantly, the absence of these neutralizing antibodies to p17 correlated with rapid disease progression in AIDS patients. To determine the sites within p17 responsible for this, twelve monoclonal antibodies to p17 were made. A infected human monocytoid leukemic cell line, THP-1, and a neoplastic T-cell line, Hut 78, reacted with the cell surface of HIV-infected cells. Of two p17 antibodies (p17A and p17B) which inhibited viral infectivity in vitro, only one (p17A) reacted with the cell surface of HIV-infected cells. Epitope mapping revealed that p17B recognized a peptide from 17-22 and p17A recognized two peptides from 12-19 and 100-105. Soluble synthetic peptides from 12-22 blocked virus neutralization but had no effect on binding of p17 on the cell surface by p17A, while peptides to 100-105 blocked binding. The myristilated N-terminal gag protein p17 was identified on the cell surface of HIV-infected cells. Using specific HIV-I p17 monoclonal antibodies, which neutralize cell-free viral infections, the epitopes defined by these monoclonal antibodies were identified near the amino terminus of the protein. Soluble synthetic peptides for this region completely inhibited the reactivity of the anti-p17s, thus, identifying regions on the p17 molecular necessary for virus netralization and cell surface binding. Reactivity to HIV-1 p17 may be an important component of immunity against HIV-I.

V. Preferential Suppression of Myelopoiesis in Normal Human Bone Marrow Cells Following In Vitro Challenge With Human Cytomegalovirus (CMV)

The pathogenic effects of human CMV infection in vitro on hematopoiesis were investigated. Normal human bone marrow cells from both seronegative and seropositive donors were challenged with CMV (Towne or wild-type strain) and tested for their responsiveness to the recombinant hematopoietic growth factors respectively. Regardless of the serostatus of the donor, infection with CMV resulted in a significant decrease in the proliferation and colony formation of hematopoietic progenitor cells in response to both growth factors, with more pronounced suppression in response to G-CSF being observed. Evaluation of the colony composition revealed a profound decrease in colonies of the granulocytic (CFU-G), or granulocyte-macrophage (CFU-GM) lineages, while suppression of multipotential (CFU-GEMM) and erythroid (BFU-E) colony-forming cells occurred following infection with wild-type but not the laboratory strain of CMV. Although no evidence of productive virus infection could be seen in colony-forming cells, in situ hybridization studies and immunohistochemical staining revealed the presence of CMV-specific mRNA and immediate-early antigens, demonstrating that a small proportion of cells were abortively infected. These studies demonstrate that CMV can infect bone marrow progenitor cells and interfere with normal hematopoiesis in vitro, which may help to explain the hematological defects seen during acute infections with CMV in vivo.

VI. Interaction of Human Cytomegalovirus (HCMV) with HTLV and HIV-infected Lymphocytes

Amongst the other human viruses also know to infect lymphoid cells are some members of the Herpes virus family, particularly HCMV. This virus is also known to cause immunosuppression, and is commonly associated with AIDS-related deaths. Nevertheless, any interaction between HCMV and HTLV or HIV remains obscure, nor is it known whether the viruses can replicate stimultaneously in the same cell. This present work was undertaken to investigate whether HCMV could infect T lymphocytes harboring the HTLV-I or HIV-I genome, and whether such superinfection with HCMV would result in an additive effect on any of characteristics of the host lymphocyte or the replication of either virus. Following challenge of HTLV-I or II-infected lymphocytes with HCMV, an increase in HCMV titer was seen which peaked at 5 days post-infection, and infectious progeny virus could still be detected between 11 and 14 days post-infection. Similarly, in HUT 78 cells, a lymphoblastoid line from a patient with Sezary syndrome, HCMV was detected up to 11 days post-infection; however, when the same cell line was infected with HIV followed by challenge with HCMV, no infectious progeny could be detected after 5 days of virus challenge, suggesting that pre-infection with HIV interferes with the persistence of HCMV in these cells. Although 5-20% of cells expressed HCMV-specific antigen as detected by staining with HCMV-specific peroxidaselabeled antibodies, only 0.01-4% of the total cell population formed infectious centers when assayed on permissive fibroblasts. In HTLV-I and HTLV-II-infected cell lines, neither cell viability and proliferation nor were there any significant differences in the biological functions or surface markers followed infection by HCMV. In contrast, both HIV-infected and noninfected HUT 78 cells showed increased Tac expression 5 days after challenge with HCMV. HCMV did not induce the production of IL 2 or IFNγ in any of the cell lines. Dot blot hybridi-zation using a 32P-labeled probe to the immediate-early region of HCMV detected the expression of viral mRNA in all cell lines except for HIV-infected cells at 14 days post-infection, again suggesting that the presence of HIV interferes with HCMV persistence. To investigate the interaction between HTLV-I and HCMV, MT-2 cells were infected with HCMV and transfected with a chimeric plasmid construct consisting of the LTR sequences derived from a molecular clone of HTLV-I fused to a bacterial CAT. We observed enhancement of the HIV-LTR directed expression of CAT by HCMV. These results demonstrate that HCMV can indeed replicate in a small proportion of lymphocytes previously infected with human retroviruses, and transactivation of the viral genome by HCMV can occur. The interaction of HCMV with cells latently and restrictedly infected with human retroviruses may have important consequences in disease progression.

PUBLICATIONS

Papsidero L, Sheu M, Ruscetti F. Human immunodeficency type-1 neturalizing monoclonal antibodies which react with p17 core protein: Characterization and epitope mapping. J Virol 1989;63:267-72.

Brownell E, Ruscetti F, Smith R, Rice N. Detection of rel-related RNA and protein in human lymphoid cells. Oncogene 1988;3:93-8.

Loughran T, Starkebaum G, Ruscetti F. Similar rearrangements of T-cell receptor beta gene in cell lines and uncultured cells from patients with large granular lymphocytes. Blood 1988;73:1613-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09254-07 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical and Molecular Mechanisms of Growth Factor Modulated Proliferation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	W. L. Farrar, Jr.	Senior Investigator	LMI, NCI
Others:	D. F. Michiel	Visiting Fellow	LMI, NCI
	A. T. Brini	Visiting Fellow	LMI,. NCI

COOPERATING UNITS *(if any)*

National Institute of Dental Research, NIH (L. Wahl); Georgetown University, (K. Clouse).

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Cytokine Molecular Mechanisms Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

IL 2 regulates the transcription of a number of genes including the proto-oncogenes, c-fos, c-myc, c-myb as well as other genes such as ornithine decarboxylase (ODC) and the IL 2 receptor α chain (Tac). Antiproliferative signals such as cyclic AMP selectively inhibit IL 2 directed increases in c-myc and ODC mRNA accumulation. The specific deletion of c-myc protein biosynthesis by synthetic "anti-sense" oligonucleotides inhibited PHA or IL 2 directed proliferation of human T cells. IL 2 also stimulates mRNA expression and protein synthesis of ancient stress genes such as HSP-70. We have investigated the DNA binding proteins which recognize the enhancer regulatory element of the HSP-70 gene. We are likewise examining DNA binding proteins specific for the c-myc and IL 2 receptor and HIV-LTR enhancer sequences to determine what signals modulate the effects of these proteins on their respective gene transcription.

We have recently shown that hematopoietic cytokines stimulate one or more tyrosine kinases. The receptors for these cytokines lack intrinsic kinase domains but apparently couple to these protein kinases. We are characterizing the substrates of these tyrosine kinases and are examining homologous cell lines transformed with v-src family oncogenes for substrates commonly phosphorylated by the growth factor stimulation and tyrosine kinase oncogenes.

The HIV envelope protein (gp120) was shown to be a potent stimulator of monocyte function, causing the release of IL 1 and PGE2. Additionally, gp120 alters human monocyte chemotaxis and inflammatory cell function. Mediators released by monocytes, such as tumor necrosis factor (TNF) increased HIV productivity in latently infected T cell lines.

PROJECT DESCRIPTION

PERSONNEL

William L. Farrar, Jr.	Senior Investigator	LS, LMI, NCI
Dennis F. Michael	Visiting Fellow	LS, LMI, NCI
Anna T. Brini	Visiting Fellow	LS, LMI, NCI

OBJECTIVES

The laboratory has developed research programs in two areas: 1) biochemical and molecular mechanisms of lymphoid and myeloid growth, and 2) regulation of immunobiology and HIV infection by virion and cytokines.

MAJOR FINDINGS

I. Biochemical and Molecular Mechanisms of Lymphoid and Myeloid Growth

The identification of protein kinase activation and the cellular substrates of the enzymes is one of the most significant areas of growth factor and oncogene research.

We have recently initiated studies which are designed to examine the participation of tyrosine kinases in lymphokine-mediated signal transduction. Previous studies used techniques which detected the most abundant phosphorylation reactions, namely serine-threonine. We have now employed anti-phosphotyrosine affinity capture methods using antibody-linked sepharose beads to enrich for substrates phosphorylated on tyrosine residues. We have found that IL 3 stimulates tyrosine phosphorylation of a number of proteins including a 140 kd protein that we suspect is associated with the receptor.

IL 2 also regulates a different tyrosine kinase, associated with the β chain of the receptor that is involved in the activation of natural killer cells (LAK) and T cell proliferation. We are addressing whether this kinase is related to known oncogenes and are purifying the kinase to permit molecular cloning. This kinase is being compared to two other tyrosine kinases found in association with the human GM-CSF and IL 3 receptors.

Progression through the cell cycle or terminal differentiation has been associated with the expression of new genes which provide additional de novo instructions to the cells. We have examined a modest number of genes expressed by IL 2 stimulation of G_1 arrested T lymphocytes. Among genes implicated in cell cycle progression for tissues or diverse origins are the nuclear proto-oncogenes. While many of the cytosolic or membrane bound proto-oncogenes (some with kinase activity) are upregulated in G_o -> G_1, the nuclear proto-oncogenes c-fos, c-myc, and c-myb are sequentially regulated by IL 2. Cleveland et al. showed that IL 2 stimulates the transcription and steady state mRNA accumulation

of these nuclear proto-oncogenes and not of cytosolic/membrane oncogenes. Subsequent studies by Harel-Bellan and Farrar have also shown that IL 3, G-CSF and GM-CSF stimulate essentially the same program of expression. Phorbol ester also triggers much of the same gene expression as IL 2 ligand in either lymphoid or myeloid cells. Other genes such as ODC, important for cellular proliferation, are also stimulated by IL 2 or CSFs. Recently, we have observed that an extraordinarily ancient set of genes are regulated by IL 2 or CSFs. Ferris et al. have shown that IL 2 stimulates steady state mRNA and protein synthesis of a member of the heat shock protein family, HSP 70. Similarly, we have found that IL 3, GM-CSF and G-CSF regulate the expression of these proteins in myeloid cells.

One method employed to specifically delete gene products, believed to be involved in the regulation of biological responses to exogenous stimuli has utilized "anti-sense" oligonucleotides. We have used short (15 mer) oligonucleotides complementary to the second exon of c-myc to evaluate the effects on c-myc protein synthesis and mitogen induced T cell proliferation. Harel-Bellan et al. showed that anti-sense c-myc oligos inhibited c-myc protein synthesis in PHA-stimulated human T lymphocytes. The deletion of c-myc protein synthesis abrogated mitogen or IL 2 induced proliferation. We have recently employed this strategy to delete IL 2 or IL 4 lymphokine expression in autocrine helper T cell clones, proving this technique applicable to the deletion of intracellular proteins and secretory molecules. We intend to test these procedures against other gene products known to upregulate during the IL 2 directed cell cycle.

The effects of IFNγ on gene regulation stimulated by CSFs were examined. Harel-Bellan et al. has shown that IFNγ did not inhibit c-myc gene transcription but c-myc mRNA accumulation was severely impaired. The studies demonstrated that IFNγ inhibited the maturation of nuclear precursor c-myc RNA into mature mRNA. IFNγ treatment of CSF-dependent myeloid cells inhibited the intron splicing process unique to c-myc precursor RNA. On the other hand, IFNγ inhibited the transcription of another proliferation-associated gene, ODC, as does cAMP in lymphoid and myeloid cells. These studies have allowed us to examine the sites of molecular inhibition at which specific anti-growth signals act. We intend to examine these phenomenon in growth factor responsive and unresponsive malignant homologous cell lines.

II. Regulation of HIV Expression by Cytokines

We have shown that the envelope protein of HIV tightly binds the CD4 cell surface receptor on human monocytes and lymphocytes. Human monocytes are activated by this process to produce monokines such as IL 1 and TNF as well as prostaglandins. The chemotactic response of monocytes to inflammatory ligands is also diminished by treatment with gp120, suggesting chemotactic receptor desensitization. Therefore, the envelope protein of HIV may alter the biological response of target cells independent of productive infection.

One monocyte-derived cytokine, TNF, was shown to stimulate HIV production in a latently infected T cell line. The regions of the HIV-LTR responding to the cytokine signal contains a regulatory sequence also found in the IL 2 receptor α

gene 5' flanking sequence. Thus, we have identified a DNA binding protein common to a cellular gene and the HIV-LTR. We have purified this protein as a 41 kDa band and are preparing rabbit anti-sera to this DNA binding protein.

The activation of this DNA binding protein may occur by independent intracellular mechanisms. One is controlled by an enzyme called protein kinase C, the other by mechanisms currently under study.

PUBLICATIONS

Farrar WL, Vinocour M, Cleveland JL, Harel-Bellan A. Regulation of ornithine decarboxylase activity by IL 2 and cyclic AMP. J Immunol 1988;141:967-71.

Ferris DK, Harel-Bellan A, Morimoto RI, Welch WJ, Farrar WL. Mitogen and lymphokine stimulation of heat shock proteins in T lymphocytes. Proc Natl Acad Sci USA 1988;85:3850-4.

Bancu AC, Cherman M, Sulica AI, Goto T, Farrar WL, Herberman RB. Regulation of human natural cytotoxicity by IgG. II. Cyclic AMP as a mediator of monomeric IgG-induced inhibition of natural killer cell activity. Cell Immunol 1988; 114:246-56.

Harel-Bellan A, Brini A, Farrar WL. Gamma interferon inhibits c-myc gene expression by impairing the splicing process in a colony stimulating factor dependent murine cell line. J Immunol 1988;141:1012-7.

Farrar WL, Harel-Bellan A, Ferris DK. Characterization of CD4 glycoprotein determinant-HIV envelope protein interactions: Perspectives for analogue and vaccine development. In: Atassi MZ, ed. Critical Reviews in Immunology. Florida: CRC Press, Inc, 1988;8:315-39.

Ferris DK, Martensen T, Farrar WL. Interleukin 3 stimulation of tyrosine kinase activity in FDC-Pl cells. Biochem Biophys Res Comm 1988;154:991-6.

Farrar WL, Ferris DK, Harel-Bellan A. The molecular basis of cytokine action. In: Kuo JF, ed. Critical Reviews in Pharmalogical Sciences. Florida, CRC Press, Inc, 1988;30:372-80.

Harel-Bellan A, Durum S, Abbas A, Farrar WL. Specific inhibition of lymphokine biosynthesis using antisense oligonucleotides in TH1 and TH2 helper T cell clones. J Exp Med 1988;168:2309-18.

Farrar WL, Harel-Bellan A, Ferris DK. Biochemical and molecular events controlled by lymphokine growth factors. In: Gunn ER, ed. Cell Physiology of Blood. New York: Rockefeller Univ Press, 1988;30:372-80.

Farrar WL. Hemopoietic cytokines in brain. Prog Neuroendroc Immunol 1988; 1:18-9.

Farrar WL, Evans SW, Harel-Bellan A, Ferris DK. Molecular events associated with the action of hemopoietic growth factors. J Cell Sci 1988;10:243-56.

Ferris DK, Brown J, Martensen T, Farrar WL. IL 3 and phorbol ester stimulate tyrosine phosphorylation of unique and overlapping substrate proteins, FEBS Lett 1989;246:153-58.

Clouse KA, Powell D, Washington I, Kovacs J, Poli G, Farrar W, Barstad P, Fauci AS, Folks TJ. Monokine regulation of HIV-1 expression in a chronically infected human T cell clone, J Immunol 1989;142:431-39.

Harel-Bellan A, Farrar WL. Regulation of proliferation in a murine CSF-dependent cell line: Super induction of c-fos by the growth inhibitor 8-Bromo-cAMP, J Cell Biochem 1989;38:145-53.

Farrar WL, Vinocour M, Hill JM. Localization of interleukin 3 mRNA in mouse brain by in situ hybridization histochemistry, Blood 1989;73:137-40.

Harel-Bellan A, Brini AT, Ferris DK, Farrar WL. Modulation of the expression of a human HSP-70 CAT construct using a double stranded synthetic regulatory element as a competitor in a human T cell line. In: Kaplan G, ed. The Cellular Basis of Immune Modulation. New York, Alan R Liss 1989;55-58.

Wahl L, Corcoran ML, Arthur LO, Pyle S, Harel-Bellan A, Farrar WL. HIV envelope protein (gp120) induction of human monocyte arachidonic acid metabolites and interleukin 1, Proc Natl Acad Sci USA 1989;86:621-25.

Farrar WL, Ferris DK, Harel-Bellan A. The role of kinase activation and gene expression in the action of interleukin 2. In: Hadden J. ed. Advances in Immunopharmacology. New York, Pergamon Press, 1989;205-209.

Farrar WL, Harel-Bellan A. Regulation of ornithine decarboxylase activity by colony stimulating factors and gamma interferon, Blood 1989;73:1468-75.

Harel-Bellan A, Mishall Z, Willette-Brown J, Farrar WL. Use of fluorescienated human IL 2 to detect low and high affinity IL 2 binding sites, J Immunol Methods 1989;119:127-33.

Harel-Bellan A, Durum S, Muegge K, Abbas A, Farrar WL. Specific inhibition of lymphokine biosynthesis and autocrine growth using antisense oligonucleotides in TH1 and TH2 helper T cell clones. In: Bertaglio J, Fadelezi D, eds. Lymphokine Receptor Interactions. New York, Raven Press, 1989;81-90.

Brini AT, Harel-Bellan A, Korner M, Farrar WL. Detection of enhancer binding proteins recognizing the HIV-1 long terminal repeat by in situ gel retardation, Biochem Biophys Res Comm 1989;160:268-75.

Farrar WL, Harel-Bellan A, Ferris DK. Lymphoid growth factors: Gene regulation and biochemical mechanisms of action. In: Cohen S, ed. The Role of Lymphokines in the Immune Response. Florida, CRC Press, in press.

Farrar WL, Beckner SK, Evans SW, Brini AT, Ferris DK, Harel-Bellan A. Regulation of phosphorylation and gene expression in cytotoxic lymphocytes by interleukin 2. In: Herberman R, Lotzova E, ed. The Role of IL 2 and IL 2 Activated Killer Cells in Cancer. Florida, CRC Press, in press.

Harel-Bellan A, Rimsky L, Krieff C, Farrar WL, Mishall Z. Flow cytometry resonance energy transfer suggests an association between low affinity IL 2 binding sites and HLA class I molecules. EMBO J, in press.

Farrar WL, Korner M, Brini AT, Harel-Bellan A, Ferris DK. Hemopoietic growth factor signal transduction and regulation of gene expression. In: Dexter M, Garland J, ed. Molecular Hematology, in press.

Birchenall-Sparks M, Farrar WL, Ruscetti F. The role of IL 2, IL 3 and phorbol esters in signal transduction. Exp Hematol, in press.

Farrar WL, Korner M, Brini AT. The role of calcium in cellular proliferation. In: Anghileri L, ed. The Role of Calcium in Biological Systems. Florida, CRC Press, in press.

Farrar WL. Evidence for the common expression of neuroendocrine hormones and cytokines in the immune and central nervous systems. Brain, Behavior and Immunity, in press.

Ferris DK, Willette-Brown J, Linnikin D, Farrar WL. Comparative analysis of IL 2 and IL 3 induced tyrosine phosphorylation. Lymphokine Res, in press.

Wahl SM, Allen JB, Gartner JM, Orenstein DE, Chenoweth, Popovic M, Arthur LO, Farrar WL, Wahl LM. Human immunodeficiency virus and its envelope glycoprotein down regulates chemotactic ligand receptors and chemotactic function of monocytes. J Immunol, in press.

Farrar WL, Ferris DK, Linnekin D. Hematopoietic growth factor regulation of protein kinases and consensus proliferation genes. In: Metcalf D, ed. Molecular Control of Hemopoiesis. United Kingdom, John Wiley & Sons, in press.

Farrar WL, Ferris DK, Michiel DF, Linnekin D. Induction of protein phosphorylation during leukocyte activation. In: Rotstein O, Grinstein S, eds. Current Topics in Membrane and Transport on Mechanisms of Leucocyte Activation. New York, Academic Press, in press.

Harel-Bellan A, Brini AT, Ferris DK, Farrar WL. Binding of a high affinity protein to a synthetic enhancer element in situ modulate regulation of the HSP promoter. Nuc Acids Res, in press.

Ferris DK, Willette-Brown J, Ortaldo JR, Farrar WL. IL 2 regulation of tyrosine kinase activity is mediated through the p70-75 beta chain of the IL 2 receptor. J Immunol, in press.

Farrar WL, Ferris DK. Two-dimensional analysis of IL 2 regulated tyrosine kinase activation mediated by the p70-75 beta chain receptor. J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09264-07 LMI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Normal and Neoplastic Hematopoietic Cell Growth: Role of BRMs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. W. Ruscetti	Senior Investigator	LMI, NCI
Others:	M. C. Birchenall-Sparks	Guest Researcher	LMI, NCI
	L. A. Falk	IRTA Fellow	LMI, NCI
	C. M. Dubois	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

LEI, BRMP, DCT, NCI (H. Gooey, R. Wiltrout); LMI, BRMP, DCT, NCI (J. Oppenheim); OAD, BRMP, DCT, NCI (D. Longo); PRI, NCI-FCRF (W. Urba, J. Rossio, J. Keller, J. Kasper, J. Mikovits, K. Sill); Oncogen, Inc. (D. Twardzik, T. Purchio) Collagen, Inc. (L. Ellingsworth).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Lymphokine Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.75 .75	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In studying humoral regulation of hematopoietic cell growth, we have found that transforming growth factor β has potent bifunctional effects. TGF β1 and TGF β2 are equipotent selective inhibitors of hematopoiesis that halt the growth of early human and murine progenitors but not more differentiated progenitors. Using purified murine hematopoietic stem cells in single cell assays with IL 3, it was shown that TGF β acted directly on the cells to block growth. The earliest measurable events in vitro such as high proliferative potential colony formation (HPP-CFU) and induction of Thy-1 antigen were also inhibited. In erythroid development, CFU-GEMM and BFU-E, but not CFU-E, were inhibited. Thus, the ability of TGF β to block hematopoietic cell growth depends on the differentiated state of the cell. In contrast, addition of TGF β to murine GM-CSF stimulated marrow cells greatly augmented growth. This increase was primarily in the granulocyte compartment. Bone marrow cells 48 hrs after 5-fluorouracil marrow treatment contain a progenitor cell whose growth absolutely depended on GM-CSF and TGF β. In vivo experiments with TGF β show the same effects on hematopoiesis as the in vitro. In contrast to myeloid leukemic cells which were sensitive to TGF β inhibition, cultured lymphoid leukemic cells were insensitive to TGF β, whereas normal lymphoid cells are sensitive to TGF β. Functional TGF β receptors leading to growth inhibition could be induced on these cells by PMA. Thus, the loss of negative regulation by TGF β may play a role in the growth of some leukemic and malignant lymphoid cells.

PROJECT DESCRIPTION

PERSONNEL

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Maria C. Birchenall-Sparks	Guest Researcher	LS, LMI, NCI
Lydia A. Falk	IRTA Fellow	LS, LMI, NCI
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MAJOR FINDINGS

I. Transforming Growth Factor β: A Selective Growth Inhibitor for Hematopoietic Progenitor Cells

In general, two techniques have been used to study hematopoietic cell growth and differentiation in vitro: (1) the measurement of cell number and type in suspension culture and (2) colony formation by different cell types in semi-solid media. Both assays depend on adding exogenous humoral factors for growth stimulation. Factors used here include recombinant murine GM-CSF, recombinant human G-CSF and CSF-1 (M-CSF), which induce terminal maturation of the cell types for which they are named, and erythropoietin (Epo), which induces end-stage erythroid maturation. Also, IL 3 which induces multipotential bone marrow colony formation (CFU-GEMM) consisting of granulocytes, erythroid cells, monocytes and megakaryocytes, was used. TGF-β1 is a potent inhibitor of IL 3 induced murine bone marrow proliferation and colony formation but, surprisingly, has little or no effect on the growth and differentiation induced by GM-CSF, G-CSF or CSF-1. TGF-β1 only inhibits early erythroid differentiation which is stimulated by Epo in combination with other factors in the GEMM or BFU-E assay while Epo-induced terminal erythroid differentiation (CFU-E assay) is unaffected. Also, IL 3 but not GM-CSF induced granulocyte-macrophage colonies were inhibited by IL 3. However, small clusters (5-20 cells) of terminally differentiated pure myeloid colonies were consistently seen in cultures containing IL 3 and TGF-β1. These results suggest that TGF-β1 selectively inhibits early murine hematopoietic progenitor growth and differentiation but not more mature progenitors. These inhibitory effects of TGF-β are at least partially reversible and non-toxic, since cell viability was not effected and colony formation was restored by 50% by washing TGF-β1 out after 48 hrs. Antisera to the N-terminus of TGF-β1 completely abrogated the inhibitory effects of TGF-β1 on hematopoiesis but had no effect on the identical inhibitory effects of TGF-β2.

We next sought to extend these observations on the effect of TGF-β to the proliferation of normal human bone marrow. In contrast to the mouse, both human IL 3 and GM-CSF, in the presence of Epo, promoted the growth and differentiation of multipotent as well as erythroid BFU-E colonies in vitro. In the presence of TGF-β1, only pure small clusters of either differentiated granulocytes or monocytes were observed. Therefore, TGF-β1 blocks the growth and differentiation of early human hematopoietic progenitors while allowing clusters (less than 40 cells) of more differentiated progeny to develop. Also, GM colony

formation induced by IL 3 and GM-CSF was inhibited by TGF-β1. However, as in the mouse, G-CSF induced colony formation was not inhibited by TGF-β. Removal of T-cells, monocytes and other adherent cells from bone marrow did not alter these results suggesting TGF-β1 was acting directly on the target cell. In our hands, TGF-β1 and TGF-β2 had identical effects on inhibiting hematopoietic cell growth and colony formation at similar doses in all these assays. Therefore, TGF-β1 and TGF-β2 suppress the growth and differentiation of early growth factor-mediated events but are inactive on late acting growth factor-mediated myeloid and erythroid responses.

II. Primitive Hematopoietic Progenitor Cell Growth: Effect of TGF-β

Highly purified populations of murine hematopoietic progenitor cells were obtained by either separating lineage negative (Lin-) Thy-1+ cells using monoclonal antibodies and fluorescent activated cell sorting or by allowing Thy-1 negative bone marrow cells to become Thy-1 positive by culturing in the presence of IL 3 and then purified by sorting on a fluoresence activated cell sorter. IL 3 can then induce CFU-GEMM and CFU-GM colony formation by purified progenitors which are inhibited by TGF β whereas formation of CFU-M and CFU-G colonies are not inhibited. Thy-1 negative cells must become Thy-1 positive induced by IL 3 before they can form colonies of any lineage in response to IL 3. TGF β inhibits the ability of Thy-1 negative cells to become positive. The purified Lin-Thy-1+ cells were plated as single cells in Terasaki plates with IL 3. In these cultures, 1 out of 6 cells proliferated. With TGF β, 1 out of 15 cells proliferated showing that 60% of the cells were inhibited and proving a direct effect of TGF β. Stem cells surviving a single injection of 5-fluorouracil are more primitive than the average hematopoietic stem cell. An assay has been developed to measure this high proliferative potential colony forming cell. The assay requires a synergistic activity (IL 1) which acts on these cells enabling them to respond to either CSF-1 or IL 3, probably by stimulating an increase in specific receptors. The expansion of either of these stem cells with HPP-CFU potential is inhibited by TGF β.

III. Bidirectional Effect of Transforming Growth Factor β on Colony Stimulating Factor-Driven Proliferation of Purified Hematopoietic Stem Cells.

TGF β1 and TGF β2 inhibit the growth of immature hematopoietic progenitor cells including interleukin 3-induced multi-lineage colonies and hematopoietin 1 (IL 1)/colony stimulating factor 1-induced high proliferative potential colonies while more mature lineage restricted progenitors, CFU-G, CFU-M and CFU-E are unaffected. In contrast, TGF β1 promotes a 3-5 fold increase in the number of granulocyte/macrophage-colony stimulating factor (GM-CSF)-stimulated bone marrow colonies in soft agar. Also, TGF β1 increases the frequency of progenitor cells that respond to GM-CSF in limiting dilution analysis assays. In addition, a progenitor cell is present in bone marrow recovered from mice after a two day treatment with 5-fluorouracil that responds only to the combination of GM-CSF and TGF β1. The morphology and histochemistry of cells grown with GM-CSF plus TGF β1 show a marked increase in neutrophilic differentiation. To determine whether the action of TGF β1 was direct, Thy-1-positive lineage-negative (Thy-1+ lin-) progenitor cells (.1-.2% of normal marrow) were purified from bone marrow by established techniques that remove accessory cells, T cells and mature

myelomonocytic cells. These cells were seeded in culture at one cell per well in Terasaki plates. TGF β1 inhibited the IL 3-induced growth of Thy-1+ lin- bone marrow cells, but promoted the growth of GM-CSF-Thy-1+ lin- bone marrow cells. Thus, TGF β1 is a direct bifunctional mediator of hematopoietic cell growth that is dependent on the nature of the CSF and target cell present.

IV. Regulation of Hematopoietic Tumor Cells by TGF β: Possible Role of TGF β Receptors

Since escape from the action of negative regulators such as TGF β could play an important role in the growth of malignant cells, we then examined the effects of TGF β on myeloid and lymphoid leukemic cells. As previously shown, TGF β acts as an important negative modulator lymphoid cell growth. Surprisingly, it was not inhibitory for the growth of any of mature human T and B lymphoma cell lines or virally-transformed T cell lines. In a small number of fresh lymphoma cells, some were inhibited and other were not. These results suggested escape from negative regulation could play a role leukemic lymphoid cell growth. However, all the meyloid cell lines with the exception of HL-60 that were examined remained sensitive to TGF β. Since cell lines growing continuously in culture may not be truly respresentative of freshly isolated malignant cells, we examined the effect of TGF β on fresh tumor specimens. In general, growth factor responsive leukemic cell progenitors from patients with acute (AML) and chronic (CML) meylogeneous leukemia were approximately 5-10 fold more sensitive to inhibition by TGF β than cells from normal donors. Nevertheless, a small population of these cells remains insensitive to TGF β inhibition, since the degree of proliferation at maximum inhibition is still above that of background inhibition in the absence of colony stimulating factor. Unlike normal BM, the G-CSF induced proliferation of CML and AML cells was also sensitive to TGF β inhibition. To further study the effects of TGF β on leukemic cell populations, number of cell lines of different lineages were tested for sensitivity to TGF β. In all cases tested, TGF β1 inhibits the growth of IL 3 induced murine myeloid cell lines such as FDC-P1 and DA-1, regardless of their derivation. The effective dose that resulted in 50% of maximal stimulation (ED-50) was similar for all cell lines. In addition to responding to IL 3, one cell line, NSF-60, proliferates in response to GM-CSF, CSF-1, G-CSF, IL 4 and IL 6. Growth factor-induced proliferation of NFS 60 is also inhibited by TGF β1 in a dose-dependent manner with ED-50's of 6-10 pM. Since normal marrow cell growth and differentiation stimulated by GM-CSF and G-CSF were not inhibited by TGF β, these results with NSF-60 suggest that the state of differentiation of a cell and not the growth factor responsiveness determines whether TGF β will have an inhibitory effect. Human cell lines of both myeloid and monocytic lineages were sensitive to TGF inhibition, although there were also lines such as HL-60 and K562 which were insensitive.

Cross-linking studies with ^{125}I-TGF β1 and ^{125}I-TGF β2 showed that connective tissue cell types have three TGF β receptor species (280 kD, 85 kD and 65 kD). Studies of the TGF β receptors present on leukocytes reveal several differences. Few TGF β receptors are found on resting leukocytes and they are upregulated following IL 3, GM-CSF or IL 2 stimulation, presumably preparing cells for subsequent negative growth regulation. Although, these leukocytes possess predominantly the 65 kD TGF β receptor but not the 280 kD receptor. In studies on leukemic cells, most myelomonocytic cells are inhibited by TGF β while

erythroid or lymphoid leukemic cell lines are not. The growth of NSF-60, a murine meyloid cell line that responds to IL 3, GM-CSF, G-CSF, IL 4, CSF-1 and IL 6 is inhibited by TGF β regardless of the factor used. In contrast, four lymphoid leukemic cell lines and a myeloid line, HL-60, that are insensitive to TGF β have little or no TGF β receptors on the cell surface. TGF β receptors can be induced on HL-60 cells with subsequent growth inhibition. Studies with fresh human myeloid and lymphoid leukemic cells show the same pattern of TGF β inhibition. The corresponding fresh cells from which the lymphoid leukemic cell lines were developed also lack TGF β receptors. These results show that TGF β is a negative regulator of the growth of leukocytes which have a novel TGF β receptor cell surface expression. Preliminary evidence suggests that loss of TGF β receptor may play a role in leukemogenesis.

HL-60, a human mpomyelocytic leukemic cell line, can undergo terminal granulocyte differentiation following treatment with retinoic acid (RA). Since TGF β is an antiproliferative and differentiating agent, we examined the role of TGF β in RA-induced differentiation of HL-60 cells. Seven day RA treatment of HL-60 cells (1.0-100 nM) resulted in an increase in TGF β steady-state mRNA expression. Moreover, chemical cross-linking analysis using ^{125}1-TGF β showed that RA, in a dose-dependent manner, increased the expression of the 65 kD TGF β binding protein. Maximal increase in receptor expression predominantly in the 65 kD binding species was obtained with 10 nM RA. TGF β alone had no effect and suboptimal concentrations of RA (1.0 nM) had limited effect on HL-60 proliferation or differentiation. Treatment with 1.0 nM RA and subsequent addition of TGF β resulted in a marked reduction in proliferation with no corresponding increase in differentiation as determined by morphological and cytochemical analysis. Thus, RA can increase TGF β mRNA as well as upregulate TGF β receptor expression permitting an antiproliferative response to TGF β. These data suggest that RA stimulates a TGF β-mediated autocrine antiproliferative loop, but that the differentiative effects of RA on HL-60 cells is not mediated by TGF β.

TGF β1 inhibits the proliferation of a variety of hematopoietic cell lines which also produce TGF-β1 mRNA and secrete proteins suggesting autocrine growth inhibition. Two growth factor independent 32-D cell lines were established by infection of IL 3 dependent myeloid precursor cells with retroviruses containing the src, and fms oncogenes. These oncogene-transformed 32-D cell lines remain acutely sensitive to TGF β inhibition suggesting that TGF β inhibition is not affected by single pathways that these onocogenes constitutively stimulate. In an attempt to understand mechanisms involved in growth inhibition we studied the regulation of TGF β1 gene expression in these cells. TGF β1 mRNA expression has been reported to increase after TGF β1 treatment of normal and transformed cells. Here, we report that TGF-β1 can also inhibit the steady state levels of its own mRNA. Time course analysis of TGF β1 mRNA expression in the 32-D src and the 32-D fms cells treated with TGF β1 showed that after 24 hr TGF β1 mRNA levels are significatnly inhibited and this inhibition is still observed 48 hr later. These results demonstrate that: 1) transformation with tyrosine kinase oncogenes can result in growth factor independent cell lines that remain responsive to TGF β1; 2) the steady state levels of TGF-β1 mRNA decrease in oncogene transformed cell lines treated with TGF β1. Moreover, these observations suggests that loss of TGF β1 responsiveness in several tumor cells is not due to oncogene expression.

V. Regulation of Gene Expression During TGF β Inhibition of Cell Proliferation in A Myeloid Cell Line

TGF β1 is a potent immunoregulatory peptide that plays a critical role as a negative regulator of early hematopoietic cell growth and differentiation. At concentrations of 625 pg/ml TGF β1 inhibits the proliferation of the murine myeloid cell line 32D. Consistent with this observation is the expression of the TGF β1 65 kD type 1 receptors in these cells. In an attempt to understand the biochemical pathways that cause inhibition of cell proliferation, we have studied gene regulation by TGF β1 in the 32D cells. Treatment with TGF β1 decreased c-myb and ornithine decarboxylase (ODC) mRNA levels in these cells, with maximum inhibition observed at 24-48 hr. Interestingly, TGF β1 inhibited histone H2a but not H4 mRNA accumulation at 24 and 48 hr after treatment, while actin mRNA levels were unchanged. TGF β1 also down regulated accumulation of its own message; however, this modulation was not observed at the transcriptional level. In summary, genes such as c-myb and ODC, which are normally rapidly up regulated in response to growth factor stimulation, are down regulated at later time points in cells treated with TGF β1. Genes whose expression is tightly coupled to DNA synthesis, such as histones, are selectively inhibited by TGF β1. Finally, TGF β1 decreases the steady state levels of its own message, apparently at the post-transcriptional level.

VI. Gamma-Interferon Induced Fresh Human Monocytes Activate Latent Transforming Growth Factor β1

TGF β is secreted from nearly all cells in a biologically inactive form which is unable to bind receptors or recognize TGF β antibodies. Since most cells have TGF β receptors, activation of "latent TGF β" must serve as a major regulatory mechanism. In this study, we asked whether cell association was involved in activation of TGF β. The "latent" recombinant complex used contains one dimeric TGF β1 molecule noncovalently associated with a disulfide-bonded dimeric remnant of the precursor. Fresh human monocytes stimulated by gamma-interferon, activates "latent" recombinant TGF β1, in a dose dependent amnner. The activated TGF β1 released into the media is neutralized by a TGF β1 monoclonal antibody and has a mass (24 kD) identical to native TGF β1. Approximately 50% of acid labile material is activated by monocytes in 8-12 hr. Untreated monocytes, gamma-interferon alone or cell-free supernatants from monocytes have no effect. Thus, TGF β1 activation by monocytes probably requires gamma-interferon mediated gene expression as well as a cell-associated processing event.

VII. Inhibition of Early Murine Hematopoietic Progenitor Cell Proliferation Following In Vivo Locoregional Administration of TGF β1

Transforming growth factor β1 (TGF β1) has been shown in vitro to be a potent negative regulator of growth and differentiation of early hematopoietic progenitor cells, but not of more mature progenitors. However, little information is yet available regarding similar effects in vivo. We have developed an approach whereby TGF β1 can be administered locoregionally to the bone marrow via direct injection into the femoral artery. Our studies show that intrafemoral administration of a single bolus dose of TGF β1 potently inhibits the baseline and IL 3-driven proliferation of bone marrow cells. This

inhibition is relatively selective for the earlier multipotential CFU-GEMM progenitor cells since these are completely inhibited while the more differentiated CFU-C colonies are inhibited by about 50%. The inhibition of hematopoietic progenitor growth and differentiation is both time and dose dependent with the maximal effect on the marrow observed at 24 hr with doses $\geq$ 5 μg/mouse, and the effect is reversed at later times. A possible practical implication of these in vivo results could be the use of TGF β1 to protect stem cells in the bone marrow from the myelotoxic effects of chemotherapeutic drugs.

PUBLICATIONS

Young H, Birchenall-Sparks M, Ruscetti F. Characterization of interleukin 2 and phorbol myristate acetate augmentation of transfected human gamma interferon genomic DNA. J Interferon Res 1988;8:527-38.

Takacs L, Ruscetti F, Kovacs E, Rocha B, Brocke S, Diamantstein T, Mathieson B. Immature, double negative (CD4-,CD-8) rat thymocytes do not express IL 2 receptors. J Immunol 1988;141:3810-8.

Loughran T, Starkebaum G, Ruscetti F. Similar rearrangements of T cell receptor β gene in cell lines and uncultured cells from patients with large granular lymphocyte leukemia. Blood 1988;72:613-5.

Sing G, Keller J, Ellingsworth L, Ruscetti F. Transforming growth factor-beta selectively inhibits normal and leukemic human bone marrow cell in vitro. Blood 1988;72:1504-11.

Keller J, Mantel C, Sing G, Ellingsworth L, Ruscetti S, Ruscetti F. Transforming growth factor-beta selectively inhibits early murine progenitor cells and the growth of IL 3 dependent myeloid leukemia cell lines. J Exp Med 1989;168:737-49.

Keller JR, Sing GK, Ellingsworth LR, Ruscetti FW. Transforming growth factor B: Possible roles in the regulation of normal and leukemic hematopoietic cell growth. J Cell Biochem 1989;39:107-15.

Talmadge J, Schneider M, Keller J, Ruscetti F, Longo D, Pennington R, Bowersox O, Tribble H: Myelostimulatory activity of recombinant human interleukin 2 in mice. Blood 1989;73:1458-67.

Sing GK, Keller JR, Ellingsworth LR, Ruscetti FW. Transforming growth factor B1 enhances the suppression of human hematopoiesis by tumor necrosis-α or interferon-α. J Cell Biochem, 1989;39:174-85.

Goey H, Keller J, Back T, Longo D, Ruscetti F, Wiltrout R: Inhibition of early murine hematopoietic progenitor cell proliferation after in vivo locoregional administration of transforming growth factor β1. J Immunol, in press.

Faltynek CR, Princler GL, Ruscetti FW, Birchenall-Sparks M. Lectins modulate the internalization of recombinant interferon-alpha and the induction of 2'5'-Oligo(A) synthetase. J Biol Chem, in press.

SUMMARY REPORT

LABORATORY OF EXPERIMENTAL IMMUNOLOGY

October 1, 1988 through September 30, 1989

INTRODUCTION

The Laboratory of Experimental Immunology (LEI) of the Biological Response Modifiers Program (BRMP), Division of Cancer Treatment (DCT) was formed in 1985 as a result of the reorganization of the existing Biological Therapeutics Branch (BTB), with Dr. John R. Ortaldo as the Laboratory Chief. The LEI is composed of three sections: the Cellular and Molecular Immunology Section (CMIS), Dr. Howard A. Young, Head, who replaced Dr. Craig W. Reynolds after his departure in 1988; the Leukocyte Differentiation Section (LDS), Dr. John R. Ortaldo, Acting Head; and the Experimental Therapeutics Section (ETS), Dr. Robert H. Wiltrout, Head.

The LEI conducts studies on biological response modification and the application of these studies to the therapy of cancer. Indepth studies are performed on cell-mediated immune effector mechanisms, molecular biology of lymphokine gene structure and expression, lymphokine biological activities, monoclonal antibodies (MAb), growth factors, and other host responses that may be useful for cancer treatment. Selected biological response modifiers (BRMs) are studied for their effects on the immune system and other aspects of host responses, with a particular focus on the therapeutic implications of such effects. Based on such information, protocols for therapy of tumors in experimental animals and in cancer patients are developed and studies are performed to evaluate the therapeutic efficacy of selected BRMs.

LEUKOCYTE DIFFERENTIATION SECTION

The Leukocyte Differentiation Section (Dr. John Ortaldo, Acting Head) studies differentiation and activation of human and murine lymphocytes and the role of the cellular immune system in mediating antitumor immune responses. The primary goal of the LDS is to use existing MAb probes and to develop new MAb for analyzing the differentiation and activation of hematopoietic cells. The focus of this group is to identify and manipulate immunologically important leukocyte subsets in normal and disease states. The specific aims of the LDS are: 1) to conduct basic and applied research regarding the natural immune system in humans and experimental animals; 2) to study human and murine cell-mediated immunity, with emphasis on surface molecules and mechanisms of tumor cell lysis; 3) to study the control of lymphocyte activation; and 4) to study the mechanisms of tumor cell resistance. The section devotes considerable attention to the study of the recognition and post-recognition events of tumor cell lysis by CD3- large granular lymphocytes (LGLs). This lytic event can be divided into three distinct stages: 1) target cell recognition and binding; 2) release of cytolytic factor(s); and 3) target cell lysis. Although the mechanism by which natural killer (NK) cells recognize tumor cells remains incompletely elucidated, our studies have considerably enhanced the understanding of these events.

While studying surface antigens on NK-sensitive K562 tumor target cells, we have developed a MAb that blocks the binding and lysis of CD3- LGLs. An anti-idiotypic antibody has also been developed, which appears to recognize an NK antigen receptor (NK-R) and which may facilitate the identification of that receptor. This antibody reacts with an 80-100 and 160 kD protein. Consistent with the hypothesis that the antibody recognizes the NK-R, F(ab')2 fragments of the antibody: 1) react primarily with CD3- LGLs; 2) block binding and lytic functions; and 3) induce significant activation of LGL effector functions after pretreatment. We are currently attempting to produce a MAb to this 80-100 and 160 kD molecule for further biochemical characterization of the NK-R. In collaboration with Dr. John Roder (Mt. Sinai Hospital, Canada) and the CMIS, a recombinant cDNA clone, which reacts with the rabbit antibody has been isolated from a CD3- LGL library and is a potential candidate for the NK receptor gene. This cDNA sequence represents a single copy gene in both human and mouse DNA and does not have any homology to known genes.

Our previous results provide the first direct evidence that a secretory event involving these granules is associated with the lysis of both tumor cells and microbial agents. The observation that LGLs secrete numerous soluble cytotoxic factors [e.g., tumor necrosis factor alpha (TNFα), interferon (IFNα/γ)] after contact with NK-susceptible target cells provides support for the notion that NK cytotoxic activity involves a secretory event. Our efforts are particularly focused on the specificity and mechanism of action of NK cytotoxic factor (NKCF). NKCF has been compared, in terms of its production and properties, to other recombinant cytokines, including lymphotoxin (LT) and TNF. We have found that NKCF is distinct from these cytokines, because antibodies directed to IFNα, IFNγ, LT or TNF failed to neutralize the cytotoxic activity observed with NKCF preparations. To assist in the characterization and purification of NKCF, mouse MAbs that neutralize the NKCF-related cytolytic activity of rat and human LGL-derived supernatants have been developed by the LDS. It is of interest that these MAbs do not inhibit TNFα, IFNα/γ, LT or leukoregulin. The importance of the NKCF molecule in cell-mediated cytotoxicity was indicated by the observation that NK activity in rat leukocytes is inhibited following treatment with these MAbs. Biochemical analysis of labeled NKCF-containing supernatants indicate that the major protein recognized by these anti-NKCF MAbs is approximately 12,000 kD. These anti-NKCF MAbs should be very useful in further purifying and biochemically characterizing NKCF and for studying its role in various cell-mediated cytotoxicity assays.

In addition to the studies with NKCF, we have reported that CD3- LGLs produce and secrete various other cytokines, including IL-1α/β, IL-2, IFNs, colony stimulating factor (CSF) and B cell growth factor (BCGF-I), IL-4. These findings have stimulated interest in the NK cell as an immunoregulatory cell. We have demonstrated that IL-2, in addition to its role as a potent activator of cytotoxicity, induces transcription/translation of IFNγ from purified populations of CD3- LGLs. To initiate IFNγ production, CD3-/CD16+ LGLs require only a single signal with a lymphocyte-activating agent such as phytohemagglutinin, phorbol myristate acetate, IL-2 or ionomycin. In contrast, CD3+ T cells require two stimuli for high levels of IFNγ production. Present studies are being extended to examine the role of IL-1, IL-4, macrophage CSF and other biologically relevant cytokines in stimulating IFN production.

Another major focus in the section has been to study the regulation of human and murine NK activity. IL-2 treatment of LGLs dramatically broadens target cell specificity and strikingly augments the overall degree of lysis. On the basis of IL-2's role as a potent activator of cytotoxicity against fresh autologous tumor target cells, studies have been initiated to examine the progenitor and effector phenotypes of these lymphokine-activated killer (LAK) cells. The observation that treatment of LGLs with IL-2 alone increased NK activity, stimulated IFNγ secretion and promoted LGL growth in the absence of the TAC IL-2 receptor fostered the hypothesis that LGLs express an IL-2 receptor other than the TAC molecule. In support of this hypothesis, IL-2 increased the lytic capacity of LGLs and the production of IFNγ in the presence of an anti-TAC (p55) MAb. These observations contributed to the discovery of the β-chain (p75) of the IL-2 receptor, which is constitutively expressed on CD3- LGLs. Our present studies are focused on studying the regulation of CD3- LGL using antibodies to the IL-2Rβ chain. In addition, studies are proceeding to examine the signal transduction events involved both in IL-2 activation and target cell interactions.

The LDS has developed a model to further our understanding of the origin, differentiation and potential function of murine and human NK cells. In these studies, we have compared CD3- LGLs isolated from mouse liver with the CD3- dLyl thymocyte subset. Unexpectedly, studies have shown that the dLyl thymic subset can repopulate liver LGLs. Thus, the dLyl thymocyte appears to represent a population of cells that can regenerate liver LGLs as well as mature T cells. These studies are the first to link CD3- LGLs with a specific T lineage progenitor. Recently, we have generated a rat MAb (4D11) that recognizes a novel 88 kD antigen (LGL-1) on the surface of LGLs isolated from mouse liver or spleen. Immunofluorescence studies indicate that this antigen is expressed on the LGLs of all strains of mice but is not detected on thymocytes, T or B lymphocytes or other hematopoietic populations. Further studies to examine the contribution of the LGL-1 lymphocyte subset to LAK or augmented NK activity have indicated that mouse NK cells can be divided into two major subsets, NK1.1+/LGL1+, which mediate the majority of fresh NK activity and NK1.1+/LGL1-, which generate most LAK activity.

CELLULAR AND MOLECULAR IMMUNOLOGY SECTION

The Cellular and Molecular Immunology Section (CMIS; Dr. Howard Young, Head) studies the role of the cellular immune system in mediating antitumor immune responses. The general goals of this section are to use molecular approaches to: 1) investigate in detail the mechanisms by which BRMs augment and regulate natural and acquired antitumor immune responses and 2) study the mechanism(s) by which tumor cell susceptibility to biological and immunological defense systems can be enhanced. The specific aims of the CMIS are: 1) to study human and murine specific T cell-mediated immunity, with emphasis on T cell specific regulation of gene expression, utilizing IFNγ as a model system; 2) to study the control of lymphocyte activation at the molecular level; 3) to study the mechanisms of tumor cell resistance, including resistance to chemotherapeutic and immunological effector molecules.

The CMIS devotes a considerable effort towards understanding how lymphokine/cytokine gene expression is regulated at the molecular level. One experimental approach utilized to explore this question has been the analysis of the role of DNA sequences in the control of IFNγ gene expression. This gene has been chosen because expression of IFNγ is restricted to two cell types, T cells and CD3-

LGLs. Human IFNγ genomic DNA has been introduced into a number of murine cell lines as well as the mouse germline. While expression in the transgenic mouse is restricted to those cell types which express the mouse IFNγ gene, expression in the murine cell lines differs depending upon the stimulating agent utilized. In a murine T cell line, gene expression can be enhanced by phorbol esters or IL-2 while in a murine B cell line, which never expresses murine IFNγ, expression of the human transgene is enhanced by LPS or a combination of phorbol esters and calcium ionophores. Current work has focused on identifying those regions of DNA which are involved in the response to various stimuli and the data indicates that there are at least two enhancer-like elements in the human IFN genomic DNA, one of which appears to be tissue specific. The second enhancer region, which does not appear to be tissue specific, may also be involved in enhanced gene expression in response to calcium flux. While it is not yet clear why the apparent tissue specificity of the transgene in the mouse germline is not observed in tissue culture gene transfer experiments, preliminary evidence suggests that DNA methylation may represent an important mechanism for restricting developmental expression of IFNγ.

A second experimental approach towards understanding the regulation of cytokine/lymphokine gene expression has involved an analysis of the effects of a chemotherapeutic drug, flavone-8-acetic-acid (FAA). In collaboration with Dr. Robert Wiltrout (ETS), efforts within the CMIS have focused on a molecular analysis of the effects of FAA in vivo in order to elucidate the mechanisms by which it enhances IL-2 therapy of a murine kidney tumor model system developed by Dr. Wiltrout. We have determined that FAA rapidly induces expression of mRNA for a number of cytokines when total spleen RNA is analyzed. In addition, cytokine mRNA is also observed in liver non-parenchymal cell RNA soon after FAA administration. These results indicate that FAA is a very potent immunomodulatory agent and its primary antitumor action may be the result of its ability to induce cytokine gene induction. Current efforts are devoted to identifying the specific cell populations which express the cytokine mRNA in response to FAA. In addition, as FAA has now been shown to induce cytokine mRNA in spleen cells and a murine macrophage cell line in vitro, efforts will be concentrated on identifying the possible FAA receptors involved in gene induction as well as those regulatory regions of the cytokine genes which are required for enhanced gene expression in response to FAA.

The CMIS is also undertaking studies to analyze the role of lymphokines/cytokines in vivo during immunotherapy. In a recent approach, the CMIS has utilized gene transfection to address this question by the introduction of functional cytokine genes into the mouse kidney tumor cell line (Renca). In collaboration with Dr. Thomas Sayers, Program Resources, Inc. (PRI), the gene for TNF has been introduced into the Renca cell line and clones have been isolated which express different levels of TNF. These cells grow slightly slower in vitro than the parental Renca and are much more sensitive to the growth inhibitory effects of IFNγ. Studies are now underway to determine the consequences of TNF production on in vivo growth of the tumor cells, with particular emphasis on an analysis of the population of lymphocytes which traffic to the tumor and the type of immune response produced against the tumor.

In collaboration with Dr. Dan Longo (BRMP) and Dr. Neal Rosen of the Lombardi Cancer Center of Georgetown University, the CMIS has initiated a new area of investigation aimed at elucidating the complex interaction of growth factors in tumor progression. Initial efforts have focused on the generation of novel MAb

to unique regions of the receptor for Insulin-like Growth Factor I. Three peptides are currently being used as immunogens and the initial steps towards generation of MAb have been performed. At the same time, in collaboration with Dr. Thomas Sayers (PRI), we are using northern blotting techniques to determine whether important growth factors or their receptors are being expressed on renal carcinoma cell lines (Renca). These cells will then be exposed to single or multiple combinations of BRMs to determine the effect, if any, on growth factor/receptor expression. Cell growth in vitro and in vivo will then be monitored to determine the relevance of the growth factor/receptor expression to the growth of this tumor.

The CMIS has also been investigating the mechanisms by which tumor cells become resistant to cancer chemotherapeutic drugs. Initially, we have studied the glutathione S-transferases (GSTs), a family of enzymes involved in cellular detoxification and found to be over-expressed in certain drug resistant tumors and cell lines. We have produced MAb to two isoenzymes, GST-π and μ. Immunohistochemical analysis of human tissues and tumors with MAb to GST-π has provided evidence that it may be useful as a preneoplastic marker in human cervical and colon cancers. We have also established a colon and ovarian cancer model of drug resistance in a nude mouse xenograft system to the drugs, cytoxan and cisplatin. Current studies are examining the mechanisms of drug resistance in these model systems and circumvention of this resistance through the use of combinations of biologicals and anti-cancer drugs.

Additional efforts within the CMIS have resulted in the generation of a murine MAb to a peptide representative of the DNA binding domain of the fos oncogene product. This antibody detects an inducible, nuclear antigen of approximately 55 kD in FBJ-virus transformed cell line. In current studies, this antibody and an additional antibody to a second peptide (AA 359-378) have been utilized to evaluate the influence of the ras oncogene on fos or fos-like protein expression. Initial experiments in normal and virus-transformed cells indicated that antigen modulation could be analyzed in permeabilized cells using immune fluorescence flow cytometry. However, while ras transformed cells showed high constitutive fluorescence, northern blot analysis indicated relatively low levels of fos mRNA. To resolve the nature of the antigens being expressed, western blot analysis of normal and ras transformed fibroblasts was performed. These results indicate that there exists within the cell, previously unknown proteins which are, like fos, inducible and located primarily within the nucleus. Current efforts are aimed at purifying sufficient quantities of these proteins for amino acid sequencing to determine their relationship to fos and determining to what extent known control mechanisms governing fos and fos-related protein expression have been altered in these ras transformed cell line. It may then be possible to determine how ras, an oncogene known to be amplified in a number of malignancies may be controlling fos expression, an oncogene fundamentally important in the early events of cell proliferation.

EXPERIMENTAL THERAPEUTICS SECTION

The Experimental Therapeutics Section (ETS; Dr. Robert H. Wiltrout, Head) provides a focus within the BRMP for rapidly translating the newest and most promising cellular and molecular biological observations into appropriate experimental therapy models in vivo. The overall goals of the section are: 1) to determine the antitumor efficacy of BRMs and 2) to study the biological mechanisms by which these agents function.

The ETS is currently pursuing several areas of study. First, a major emphasis of the section has been to develop and utilize experimental models of chemo-immunotherapy (CIT) and adoptive chemoimmunotherapy (ACIT) for the treatment of both primary tumors and their metastases. To determine the effectiveness of CIT for tumors of various histological types located in different anatomical compartments, these studies are performed in models that produce tumors at various sites. In addition, the ETS investigates the efficacy of CIT and ACIT, with special emphasis on the nature of the responder and effector cells and the contribution of the recipient's immune system to the observed therapeutic effects. The ETS has performed extensive studies to determine the therapeutic efficacy of adoptive immunotherapy (AIT) with recombinant IL-2 (rIL-2)-stimulated lymphocytes and/or exogenous rIL-2 in combination with chemotherapeutic drugs for the treatment of murine renal cancer. It has been shown that AIT or chemotherapy with doxorubicin hydrochloride does not cure mice bearing Stage I renal cancer, while combination ACIT cures approximately 70% of these mice. Further studies have demonstrated that the therapeutic effects of adoptively transferred LAK cells occur in spite of poor localization of the actual cytotoxic effector population into the tumor. Subsequent experiments have revealed that LAK effector cells exhibit increased expression of the genes coding for several potent immunomodulatory cytokines, including TNFα and IFNγ. These results suggest that the antitumor effects of adoptively transferred LAK cells could be directly mediated by soluble cytokines or indirectly mediated by stimulation of host effector cells.

Additional CIT studies have further implicated a role for induced cytokines via the demonstration that the investigational drug, FAA and rIL-2 have synergistic antitumor effects against murine renal cancer. The contribution of FAA to this combination appears to be at least partially cytokine mediated since in collaboration with Dr. Howard Young (CMIS), we have shown that FAA potently upregulates the expression of genes for IFNγ, TNFα, and IFNα, and these cytokines can enhance the therapeutic effects of IL-2. This type of approach provides an experimental preclinical method for exploiting the antitumor effect of rIL-2 without expensive and complicated AIT administration.

Further studies have shown that recombinant murine IFNγ (rmIFNγ) and lipo-polysaccharide (LPS) can partially inhibit the growth of the retrovirus-immortalized GG2EE macrophage line in vitro, but rmIFNγ plus LPS more completely inhibit GG2EE proliferation. Interestingly, treatment of GG2EE cells with LPS results in increased expression of the TNFα and IL-1 α/β genes and the effect of LPS can be replaced by using rIL-1α and rTNFα in conjunction with rmIFNγ. Studies are in progress to determine whether rIL-1, rTNF, TGFβ and rIFNγ can be used therapeutically against GG2EE in vivo. Similarly, since GG2EE expresses the v-myc and v-raf oncogenes as a result of the immortalization of bone marrow (BM) cells by the J2 retrovirus, studies are being performed, in collaboration with Drs. Luigi Varesio, Laboratory of Molecular Immunoregulation (LMI) and Howard Young (CMIS), to determine whether transient transfection of antisense oligonucleotides will alter expression of v-myc and v-raf and therefore alter the tumorigenicity of the GG2EE cell line.

Because cytokines are often pleiotropic in their actions we have also performed studies, in collaboration with Drs. Jonathan Keller (PRI), Frank Ruscetti (LMI), and Dan Longo (BRMP) show that in vivo administration of rTGFβ1 can transiently arrest the proliferation of early hematopoietic stem cells. Subsequently, we have shown that both rIL-1α and or TGFβ1 have chemoprotective effects in vivo

against the toxic effects of cyclophosphamide (Cy). Further studies are in progress to determine the mechanisms for these effects and to determine whether this approach can be used to increase the therapeutic efficacy of chemotherapeutic drugs.

A second major area of emphasis in the ETS is to investigate the mechanism(s) by which BRMs augment natural immunity in vivo. The liver is being used as a model to study the localization of NK cells into a nonlymphoid organ. The results, to date, have demonstrated that many BRMs augment NK activity to a greater degree in the liver than in the blood and spleen. Further studies performed to determine the mechanism(s) by which BRMs augment NK activity and cause LGLs to accumulate in the liver have utilized the bone-seeking isotope strontium-89 to preferentially eliminate BM cellularity. The results of these studies suggest that much of the increase in liver-associated NK activity induced by BRMs occurs through an accumulation of NK cells newly derived from BM progenitors. The mechanism for this localization is currently also being studied and early results indicate that LGL can respond chemotactically to factors produced by hepatocytes and Kupffer cells following treatment with BRM.

The third general area of study by the ETS is focused on the effects of various BRMs on migration of leukocytes into the peritoneal cavity in vivo. In collaboration with Dr. Thomas Sayers (PRI), we have previously demonstrated that IL-1 was a potent stimulus for inducing migration of polymorphonuclear neutrophils. TNF (α and β) also showed some activity but on a protein basis, this activity was 50-100 times less potent. Synergy occurred between suboptimal amounts of IL-1 and TNF. Other cytokines tested at equivalent protein concentrations had no activity. We have extended these studies to investigate a possible role for cytokines in controlling the migration of NK cells into the peritoneum. Numbers of peritoneal NK cells were estimated using three different assays: (a) functional: lysis of YAC tumor targets; (b) phenotypic: utilization of the NK-specific MAb LGL-1 with FACS analysis; and (c) enzymatic: measurement of levels of granzyme A (BLT esterase) extracted from cells. In contrast with the previous findings for polymorphonuclear (PMN), NK cells did not accumulate in response to IL-1 or TNF but did migrate into the peritoneum after injection of IL-2 or IFNγ. IFNγ is more active when similar amounts of protein were compared. Indeed, NK migration in response to IL-2 could be inhibited by neutralizing MAb to IFNγ suggesting that IFNγ production in situ was critical for NK migration in response to IL-2. Since IFNγ itself does not appear to exhibit chemotactic properties it may induce production of endogenous chemotactic factors. Alternatively, the enhancement of IFNγ on NK cell migration may be due to effects on endothelial cell binding that must occur prior to leukocyte infiltration into tissue. Mechanisms underlying effects of IFNγ on migration of NK cells will be further investigated. Additional studies have shown that in unstimulated rodents the vast majority of granzyme A activity is associated exclusively with NK cells. The possible application of this observation as an objective assay for detection and quantitation of NK cells is under consideration.

Since one of the initial events in leukocyte migration in vivo involves binding to and penetration through basal lamina, Dr. Anne Pilaro has examined the interaction of specific leukocyte subsets with different basement membrane components. Isolated mouse neutrophils and peritoneal macrophages were found to bind tissue culture dishes that had been coated with laminin, collagen IV, or fibronectin. Using small molecular weight pentapeptides which bind to and inactivate either laminin or fibronectin receptors on the surface of these cells,

we were able to inhibit binding to specific substrates by up to 30% in a time and dose-dependent manner. Additionally, pretreatment of mouse neutrophils with the peptide YIGSR, which binds to the laminin receptor, was found to inhibit directed migration of neutrophils in response to either C5a or fMLP by up to 50%. Further studies are planned to examine the role of the laminin receptor in other leukocyte functions associated with emigration in vivo, such as release of lysosomal proteases or activation of the respiratory burst.

A fourth major effort of the ETS has been to develop CIT approaches for the intracavitary treatment of human colon (Ht-29) and ovarian (OVCAR-3) xenografted into nude mice. Dr. John Pearson has shown that the administration of multiple intraperitoneal treatments of human MAbs, conjugated to either Pseudomonas exotoxin or the A chain of ricin, to mice with low tumor burden resulted in highly significant increases in the life span of the treated mice. Furthermore, groups of mice that received multiple i.p. treatments of immunotoxins following cytoreductive chemotherapy exhibited a further significant increase in the mean survival time (MST) over that observed with chemotherapy or immunotoxins alone. Cells harvested from mice that showed progressive tumor growth after chemo-immunotoxin therapy exhibited no evidence of either toxin resistance or loss of antigens from the cell surface. In vitro data has shown that IFNγ and IFNα synergize with both immunotoxins, and both IFNs are currently being used in combination against Ht-29 and OVCAR-3 to further increase the therapeutic efficacy of these immunotoxins against localized peritoneal tumor growth. These results demonstrate that the combination of chemotherapeutic drugs with antigen-specific MAb conjugates can result in an additive or synergistic preclinical therapeutic effect.

The ETS is also concerned with mechanisms of BRM-induced alterations in leukocyte subsets. In this regard the fifth major area of study by the ETS focuses on the gene expression and growth factor receptors of murine thymocyte subsets. Results developed in collaboration with Dr. Kristin Komschlies (PRI) have demonstrated that the mRNA and product of the ets-1 oncogene are expressed in a 10-fold greater amount in the CD4+ (CD8-) thymocyte subset than in all other thymocyte and T cell subsets defined by CD4 and CD8. This observation suggests that the ets-1 gene product may be involved in the differentiation of the CD4+ thymocyte subset. Other work has shown that mice receiving short-term estrogen treatment are depleted of the IL-2R+ cells within the most immature subset (CD4-, CD8-, dull CD5+; dLy-1+) of thymocytes. These dLy-1+ thymocytes from treated mice are still capable of generating all other thymocyte subsets and T cells suggesting that IL-2R expression may not be a requirement for this function. Further studies examining the thymic localization of lymphoid cells as they repopulate the thymus and the regulation of T cell lineage differentiation are being pursued.

SUMMARY

Overall, the three sections of the LEI are organized to provide an integrated, complementary approach to the study of the immunomodulatory and immunotherapeutic properties of BRMs. The sections also facilitate the research efforts of other areas of the BRMP by providing expertise in cellular immunology, molecular biology and preclinical animal modeling. The ultimate goal of the LEI is to provide a focus within the BRMP for sufficiently developing immunological approaches and testable hypotheses for cancer treatment so that they can be considered for intramural and extramural clinical trials.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09256-07 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Natural Cell-Mediated Immunity: Biology and Regulation of CD3- LGL

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. R. Ortaldo	Chief	LEI, NCI
Others:	J. Frey-Vasconcells	IRTA Fellow	LEI, NCI
	A. Mason	Biologist	LEI, NCI
	W. Bere	Bio. Lab. Tech.	LEI, NCI
	J. Wine	Bio. Lab. Tech.	LEI, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., NCI-FCRF (J. Rossio); LMI, BRMP (Cytokine Mechanisms Section and Immunobiology Section); Tohoku University School of Medicine, Sendai, Japan (K. Sugamura).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Leukocyte Differentiation Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.8	0.4	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interleukin 2 (IL-2) has demonstrated a potent ability to augment NK activity and to generate killer cells against NK-insensitive targets. This IL-2-mediated augmentation appears to parallel production of IFNγ by LGL, but abrogation of antiviral activity with anti-IFNγ serum did not abolish NK boosting. These in vitro results are consistent with the hypothesis that IL-2 is the major inducer of LAK activity. In addition to NK activity, LGLs have been shown to produce a variety of lymphokines (IL-1, IFN, CSF, BCGF). A project is being conducted to investigate IFNγ gene expression and regulation in highly purified human LGLs and T cells. Within 1 hr of IL-2 treatment of freshly isolated human LGLs, IFNγ mRNA can be detected whereas active protein was secreted in the culture medium within 4-6 hrs of treatment. These results indicate that with certain stimuli LGLs may be the predominant source of IFNγ from peripheral blood lymphocytes. In addition to IL-2, specific surface proteins are being examined as potential regulatory elements. Specific monoclonal antibodies have been used to study their role in activation. The results have demonstrated that anti-CD2 monoclonal antibodies have a significant regulatory effect on CD3- LGL functions. Furthermore, preliminary findings have demonstrated that combining of selected epitopes to CD2 can result in significant synergy, as determined by NK activity and IFNγ production. CD3- LGL activities can be regulated by a variety of cytokines. In addition, the CD3- LGL represents an excellent cell type to study signal transduction leading to both modulation of cytotoxicity and gene transcription, since it represents a cell type "poised" for activation and capable of responding to a single stimulus.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Joyce Frey-Vasconcells	IRTA Fellow	LDS, LEI, NCI
Anna Mason	Microbiologist	LDS, LEI, NCI
William Bere	Bio. Lab. Tech.	LDS, LEI, NCI
John Wine	Bio. Lab. Tech.	LEI, NCI

OBJECTIVES

The objectives of this project are:

1. To study the factors regulating the activation and development of natural killer (NK) and related natural effector cells;

2. To analyze the interaction of natural effector cells with other components of the immune system;

3. To study cultures and clones of large granular lymphocytes (LGL) for their cytolytic activity and their production of soluble products in regulation of the immune system.

RESULTS

Regulation of NK Activity

A. Lymphokine Activated Killer

Our studies of progenitor cells relied on phenotypic characterization, separation on Percoll gradients, and limiting dilution analysis to determine progenitor frequency. Both CD3+ and CD3- cells were shown to generate cytotoxic cells, termed LAK effectors, from normal PBLs. Although the progenitor cell that is isolated from whole PBLs varies somewhat (depending on the target cell), the primary effector cell is coincident with the CD3- lymphocyte population on day 0, i.e., the CD16+, CD56+ activated NK cell.

Clearly, a CD3+ cell contributes a small but significant amount to rIL-2-generated cytotoxicity. However, an LGL population that is CD3-, CD8-, but bears the CD16 and CD56 markers contributes most of the rIL-2-generated LAK activity.

B. Signalling Events

One of the more interesting findings regarding IL-2 activation of human LGLs is that multiple functions are activated. IL-2 treatment of LGLs increases NK activity, induces IFNγ secretion, and promotes growth. Our results indicate that, unlike T cells, CD3- LGLs can respond to a single agent (IL-2) and express

the IL-2 receptor, as defined by TAC. Although these results were somewhat confusing, they provided some of the earliest evidence that TAC expression was not required and led to the discovery of the b-chain of the IL-2 receptor, which has been demonstrated to be constitutively present on fresh, inactivated CD3- LGLs.

Compared to fresh T cells, freshly isolated LGLs have been shown to require only a single stimulus, such as IL-2, phorbol myristate acetate (PMA), phyto-hemagglutinin (PHA), or ionophore, to produce IFNγ, while combinations of these agents had synergistic effects on IFNγ production. In T cells, in contrast, no single agent produced an adequate stimulus, while combinations of agents resulted in significant IFNγ production. When the LGL response was analyzed at the mRNA level, increased IFNγ message was detected by 60 min. These data suggest that LGLs are in a "poised" state, as demonstrated by their ability to respond rapidly to a single stimulus to transcribe and produce IFN protein.

MAbs CD8, OKT9, OKT10, CD2, CD16, CD11, and CD56, which are specific probes for analyzing leukocyte surface molecules, were used to study the potential role of these molecules in the regulation of LGL functions. Our results with the CD2 MAb and the identification of additional CD2 epitopes led us to further examine their ability to regulate NK and LAK cytotoxicity and IFNγ production. Overall, these anti-CD2s produced an approximately two-fold increase in basal LAK activity. When IFNγ production was examined, a similar trend was seen. All of the anti-CD2s except 9.6 resulted in a small increase in IFNγ production. In most experiments, the increase seen with anti-GT2 was not as dramatic. Anti-GT2 resulted in an approximately four-fold increase in basal IFNγ units, and this effect was only marginally enhanced by the addition of 9.6. In addition, anti-X11-1 again inhibited IFN production when combined with 9.6.

The conclusion drawn from these data is that MAbs against the CD2 molecule are important in regulating LGL functions. Since the natural ligand for CD2 is cell surface LFA-3, this ligand interaction delivers a signal to the cell that regulates cell functions. It is possible that CD2 promotes a down-regulation of IL-2R responsiveness, thus causing the inhibition. The antibodies may somehow signal an otherwise IL-2-responsive cell to become less responsive or nonresponsive, possibly by altering the IL-2R or changing the signal it transduces intracellularly.

Since lymphocyte receptor-ligand interactions result in intracellular triggering, it was important to examine these regulatory processes involved in CD3- LGL regulation. This is especially relevant to the CD3- LGL model, due to its ability to be triggered by single agents, e.g. IL-2, CD2, etc. In investigating the cellular regulation of these diverse functions of CD3- LGLs, we have examined the effect of PKC inhibitors on LGL function. PKC is a Ca++ and a phospholipid-dependent enzyme that is involved in membrane signal transduction. PKC activity has been shown to be important for various leukocyte functions and previous reports have suggested that PKC may be involved in regulating NK activity, including the regulation of NK cytotoxic factor release. We have utilized PKC inhibitors and a PKC activator [a synthetic diacylglycerol (OAG)], in 1) NK activity, 2) LAK activity, and 3) the expression of the IFNγ gene. We have studied the effects of 1-(5-isoquinolinesulfonyl)2-methyl-piperazine-dihydrochloride (H7), L-a-1-oleoyl-2-acetoyl-sn-3-glycerol (OAG), retinol and

staureosporin on the basal levels of NK activity and the augmentation of NK activity by IL-2 and IFN. Our finding of a dose-dependent reversal of the H7 effect, strongly supports the concept that H7's ability to inhibit NK activity is due to its effect on PKC, suggesting that PKC has a role in the expression of the NK activity.

Overall, our results suggest that PKC has a regulatory role in the expression of basal NK activity by fresh LGLs. This provides the first indication that PKC may be involved in the maintenance of a biological function rather than, or in addition to, the deliberate triggering of function by a ligand. We do not know whether the basal levels of NK activity result from a continuous in vivo exposure of the LGL to cytokines. But even if this were the case, our results indicate that PKC activity is still necessary for the expression of the NK activity that is maintained in vitro in the absence of exogenous stimulants, or that this activity is augmentable in vitro by IL-2 or IFN. In fact, we have observed that induction of the IFNγ gene is affected by PKC inhibitors. Transcription of other genes, phosphorylation of membrane and/or intracellular proteins, and metabolic activities may be equally affected. Using a panel of selective reagents that can block other cellular pathways, it should be possible to more precisely define the specific steps in which PKC activity is related to NK activity and thereby understand the regulation of these functions at the biochemical level.

PUBLICATIONS

Morgan AC Jr, Schroff RW, Ortaldo JR, Herberman RB. Occult leukocyte and tumor-associated antigens by flow cytometry, J Clin Lab Analys 1988;2:182-6.

Ortaldo JR, Longo DL. Human natural lymphocyte effector cells: definition, analysis of activity, and clinical effectiveness, J Natl Cancer Inst 1988;80:999-1010.

Ortaldo JR. Human cytotoxic effector cells: definition and analysis of activity, Immunologia Clinica 1988;67-86.

Ortaldo JR, Mathieson BJ, Wiltrout RH. Characterization and functions of natural killer cells, Ann Inst Pasteur/Immunol 1988;139:444-50.

Sakamoto S, Ortaldo JR, Young HA. Methylation patterns of the T cell receptor β-chain gene in T cells, large granular lymphocytes, B cells, and monocytes, J Immunol 1988;140:654-60.

Wiltrout RH, Ortaldo JR. New and alternative approaches to adoptive immunotherapy. Proceedings of the International Symposium on New Horizons in Tumor Immunotherapy. Fukuoka, Japan: Second Fukuoka International Symposium, 1988;in press.

Ortaldo JR. Cytokine production by CD3- large granular lymphocytes. In: Reynolds CW, Wiltrout RH, eds. Functions of the natural immune system. New York: Plenum Pub Corp, 1989;299-313.

Sakamoto S, Ortaldo JR, Young HA. Analysis of the methylation state of the T cell receptor α chain and β chain genomic DNA in human large granular lymphocytes and T cells. In: Ades EW, Lopez C, eds. Natural killer cells. Basel: Karger 1989;22-7.

Mason L, Giardina SL, Hecht T, Ortaldo JR, Mathieson BJ. LGL-1: non-polymorphic antigen expressed on a major population of mouse natural killer cells. In: Ades EW, Lopez C, eds. Natural killer cells. Basel: Karger, 1989;33-8.

Ortaldo JR, Young HA, Varesio L. Modulation of CD3- LGL functions by agonist and antagonists of PKC: effects of NK and LAK activity and production of IFN, J Immunol 1989;in press.

Wiltrout RH, Pilaro AM, Gruys ME, Talmadge JE, Longo DL, Ortaldo JR, Reynolds CW. Augmentation of mouse liver-associated NK activity by biological response modifiers occurs largely via rapid recruitment of LGL from the bone marrow, J Immunol 1989;in press.

Reynolds CW, Ortaldo JR. Nature and physiology of natural effector cells. In: Shevach E, Oppenheim JJ, eds. Immunophysiology: the role of cells and cytokines in immunity and inflammation. New York: Oxford University Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09259-07 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization and Differentiation of NK Cells in the Mouse

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. R. Ortaldo	Chief	LEI, NCI
CO-PI:	L. Mason	Biologist	LEI, NCI
Others:	R. H. Wiltrout	Head	LEI, NCI
	H. A. Young	Head	LEI, NCI

COOPERATING UNITS (if any)
Program Resources, Inc. (S. Giardina); Vaccine Research and Development Branch, NIAID, (B.J. Mathieson).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Leukocyte Differentiation Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Monoclonal antibodies (MAbs) have been generated to study the origin, differentiation, and potential function of naturally-occurring murine cytotoxic leukocytes. A rat MAb (4D11) has been generated against a novel, 87 kDa, cell surface antigen on mouse large granular lymphocytes (LGL) with natural killer (NK) activity. This antigen, LGL-1, can be detected on splenic or liver-derived LGL from most strains of mice but not on other types of lymphocytes or hematopoietic populations, and not LGL from other species. Cells electronically sorted for LGL-1 expression are highly enriched for LGL and for NK activity, whereas negatively sorted or MAb + complement-depleted LGL-1- spleen cells contained little or no NK activity. Dual-color immunofluorescence revealed subsets of LGL-1+ cells which co-expressed Ly-1 (CD5) and Thy-1, whereas CD8+ cells did not co-express LGL-1. Greater than 90% of LGL-1+ cells coexpress NK-1.1, Qa-5, and asGM1. Although LGL-1 can be found on populations of lymphokine-activated killer (LAK) cells, it has been determined that LGL-1 is not expressed on LAK precursor or effector cells. Antibody plus complement depletion of LGL-1+ cells, as well as cell sorting demonstrates that LGL-1+ cells do not contribute towards the LAK phenomenon. LGL-1 appears to split the NK cell population into two subsets, an NK-1.1+/LGL-1+ subset that can lyse YAC-1 targets and be augmented by IL-2. The remaining NK-1.1+/LGL-1- cells, although capable of lysing YAC-1 tumor targets to a somewhat lesser degree, can respond to IL-2 and develop a broadened cytotoxic response (LAK).

PHS 6040 (Rev 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Llewellyn Mason	Biologist	LDS, LEI, NCI
Robert H. Wiltrout	Head	ETS, LEI, NCI
Howard A. Young	Head	CMIS, LEI, NCI

OBJECTIVES

The major objectives of this project are: 1) to identify and characterize the cellular phenotype of leukocytes that have natural killer (NK), or lymphokine-activated killer (LAK) activity, 2) to study the origin, differentiation, development and activation of these cells in vivo and in vitro, 3) to devise new reagents that can potentially target the effects of activated leukocytes at the site of tumors in vivo.

Our specific aims are to: (a) acquire and develop a panel of MAbs and other reagents with selective or differential reactivity against mouse NK cells, (b) use MAbs to develop enrichment and selection techniques for isolation of precursors and differentiated cells from various tissues, (c) characterize the isolated precursors of augmented NK and LAK cells from spleen, liver, or bone marrow by phenotype, growth requirements in vitro and pattern of cytotoxicity on different tumor cell lines and normal lymphoid targets.

METHODS EMPLOYED

To characterize NK cells and LAK cells in the mouse, we used MAbs for the mouse that detect: 1) Ly-5, which is expressed on all leukocytes including NK cells; 2) The T cell specific antigens Ly-2 (CD8), L3T4 (CD4) and CD3 as well as Ly-1 (CD5) and Thy-1 which are also expressed on some non-T cells; 3) Qa-5, an MHC-associated, class I antigen expressed preferentially on NK and T cells; 4) NK-1.1, asialo GM1 (asGM1) and the Fc receptor for IgG, markers that have been associated with NK cells in mice and other species. MAbs and other serological reagents for detection of surface antigens were obtained from commercial sources or through collaborative agreements, titered and tested for appropriate reactivity.

I. Subset Enrichment Methods

Nylon wool nonadherent (NW-NA) cells from spleen or liver were subjected to Percoll density gradient fractionation to enrich for low density LGL. The LGL enriched fraction from spleen contains about 10-30% LGL in mice in contrast to the high level of purification seen from peripheral blood cells of humans or rats. Anti-Ig plate adherence was used to further remove contaminating B cells. Anti-Ly-2 (CD8) and anti-L3T4 (CD4) MAb were also used in indirect anti-Ig coated plate separations and facilitated complement (C)-dependent lysis to further select and/or deplete T cells. Mouse liver nonparenchymal cells that contain a

high proportion of LGL were obtained by collagenase digestion of minced perfused livers from MVE-2, Poly IC:LC, or C. parvum-treated animals. These cells were further purified by removal of NW adherent cells, Percoll density enrichment, and T cell depletion where necessary.

NK cells were enriched prior to culture in IL-2 for identification and characterization of LAK cells. LAK precursor and effector cell populations were depleted of LGL-1+ cells using antibody + C' depletion in order to determine the significance of LGL-1+ cells in the LAK response. Alternate methods to positively select the LGL-1+ and NK-1.1+ cells and determine their significance in the LAK response were performed using electronic cell sorting.

II. Cell Surface Phenotype

Phenotype of the isolated cell populations was determined by one of two methods:
a) Flow cytometry analyses (FCA) to detect and quantify cell surface antigen expression detected by immunofluorescence (IF) were performed on an Cytofluorograph and dual-color analyses were performed on a FACScan.
b) Cytotoxic elimination with MAb + C served to directly assess the phenotype of the eliminated cells relative to the level of NK or LAK function, or to eliminate unwanted or irrelevant cell subsets and to enrich LGL before IF or functional analyses.

III. MAb Production

Hybridomas producing Ig were obtained by fusion of immunized rat spleen cells with drug sensitive murine myeloma cells. After selection and expansion in vitro, ascites were produced in nude mice or MAbs were produced in vitro. Ig from ascites or culture supernatants was purified by sodium sulfate fractionation and affinity columns using protein G sepharose.

IV. Antigen Characterization

Molecular characterization of the LGL-1 antigen was performed by immunoprecipitation of surface-labeled (125-I) cell membranes with the 4D11 MAb. This precipitated material was analyzed on SDS-PAGE gels under reducing and nonreducing conditions. The extent to which the LGL-1 antigen is glycosylated was determined by digestion of solubilized immunoprecipitates with various glycosidases including neuraminidase, endoglycosidase-H, N-glycanase and O-glycanase prior to SDS-PAGE analysis.

RESULTS

Characterization of NK Cells Using MAbs

Several MAbs were generated to study the origin, differentiation, and potential function of naturally-occurring cytotoxic lymphocytes. By blocking the response to common lymphocyte antigens, rat MAbs were raised to mouse LGL. In particular, one rat MAb (4D11) was generated that recognizes a non-allelic determinant on NK-active cell populations. The 4D11 antibody recognizes a novel cell surface antigen, and expression is highly correlated with the presence of LGL in B6 mice and related congenics. IF studies indicate that this antigen is expressed on LGL

of most strains of mice but it is not detected on thymocytes, T or B lymphocytes or other hematopoietic populations. Antibody-dependent, complement-mediated lysis depletes 50 to 80% of the NK activity in leukocyte populations enriched for NK cells. Cells positively selected by electronic sorting of the 4D11+ leukocytes, contain 50 to 60% LGL and are enriched 7 to 70 fold in their lytic activity against YAC target cells. Studies indicate that the antigen recognized by 4D11 (LGL-1) is not expressed on LAK precursors or effectors. However, LGL-1 has been able to divide the NK cell population into two subsets, an NK-1.1+/LGL-1+ subset that cannot generate LAK, and an NK-1.1+/LGL-1- population that generates most splenic LAK activity.

PUBLICATIONS

Mason L, Giardina SL, Hecht T, Ortaldo J, Mathieson B. LGL-1. A non-polymorphic antigen expressed on a major population of mouse natural killer (NK) cells, J Immunol 1988;140:4403-12.

Mason L, Giardina SL, Hecht T, Ortaldo JR, Mathieson BJ. LGL-1. Non-polymorphic antigen expressed on a major population of mouse natural killer cells. In: Ades EW, Lopez C, eds. Proceedings of the 5th International Workshop on Natural Killer Cells and Host Defense. Hilton Head: 5th International Workshop on Natural Killer Cells, 1989;33-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09247-09 LEI

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Natural Cell-Mediated Immunity Mechanism of Lysis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. R. Ortaldo	Chief	LEI, NCI
Others:	J. Frey-Vasconcells	IRTA Fellow	LEI, NCI
	R. Winkler-Pickett	Microbiologist	LEI, NCI
	W. Bere	Bio. Lab. Tech.	LEI, NCI
	J. Wine	Bio. Lab. Tech.	LEI, NCI

COOPERATING UNITS (if any): Immunology Branch, Division of Cancer Biology Diagnosis, National Cancer Institute, Bethesda, MD (P. Henkart); Program Resources, Inc., Frederick, MD (J. Rossio)

LAB/BRANCH: Laboratory of Experimental Immunology

SECTION: Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION: NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.2	1.5	2.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Natural killer (NK) cells and killer (K) cells mediating antibody-dependent cellular cytotoxicity have been shown to be large granular lymphocytes (LGLs). The mechanism of cytotoxicity by LGLs can be divided into two clearly distinguishable stages. The first stage is recognition and binding of the LGLs to target cells, while the second includes post-binding events that lead to target cell lysis. Studies are proceeding to define the receptors and structures involved in NK recognition. A monoclonal antibody (MAb) was developed against NK target antigens on K562 cells and that antibody blocks LGL binding and lysis. We also developed an anti-idiotypic antibody (anti-ID) against this MAb anticipating that it might recognize the NK receptor and aid in its identification. This anti-ID antibody is reactive with an effector cell protein and blocks LGLs binding and target cell lysis. LGLs pretreated for 18 hr with the F(ab')2 anti-ID antibody significantly enhanced lysis of target cells and production of interferon-γ by LGL. It will be important to perform studies to characterize the expression of the putative receptor molecule on activated cells that have been reported to have NK activity. Further biochemical characterization of the NK receptor and its cloning are in progress. The role of soluble factors such as natural killer cytotoxic factor (NKCF) in this cell-mediated lysis has been examined to gain an understanding of the mechanism of lysis. The activity of NKCF has been divided into three distinct stages: a) production; b) binding to targets; and c) target cell lysis. The availability of procedures to independently measure these stages has enabled us to test a variety of agents that inhibit NK cell-mediated killing and to determine the factor's site of action. NKCF is produced by LGLs and its general specificity pattern is similar to that of intact NK cells. NKCF has been demonstrated to be distinct from recombinant lymphotoxin, tumor necrosis factor, and leukoregulin. We are using rat LGL cell lines as a source of NKCF and are pursuing studies leading to its molecular cloning with the aid of the specific MAb to NKCF.

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Joyce Frey-Vasconcells	IRTA Fellow	LDS, LEI, NCI
Robin Winkler-Pickett	Microbiologist	LDS, LEI, NCI
William Bere	Bio. Lab. Tech.	LDS, LEI, NCI
John Wine	Bio. Lab. Tech.	LEI, NCI

OBJECTIVES

The objectives of this project are:

1. To study natural cell-mediated immunity to tumors in man and analyze the phenotypic, biochemical, and functional characteristics of the cytotoxic cells;

2. To study, isolate and characterize the nature of NK effector cell receptors;

3. To study, isolate, and characterize the nature of NK target structures.

4. To study the nature and mechanism of cytotoxicity by LGL and to attempt to isolate and characterize soluble cytotoxic factors;

RESULTS

A. Recognition and Triggering of NK Lysis.

We have developed an IgM MAb to K562 tumor cell membrane glycoprotein, designated MAb #36. MAb #36 reacts with surface glycoprotein(s) on K562 and other NK-susceptible target cells, inhibiting binding of CD3- LGLs to target cells. It was reasoned that if this MAb reacts with a target cell molecule recognized by CD3- LGLs, then the antibody VDJ region might structurally resemble the NK-R. Therefore, an anti-idiotype antibody (anti-ID) to MAb #36 might also identify the NK-R on LGLs. The anti-ID was found to bind to human CD3- LGLs but not with other resting cells (CD3+ T cells, macrophages, neutrophils, B cells), suggesting that the molecule reacting with the anti-ID is specific for CD3- LGLs. Direct anti-ID treatment of LGLs inhibited binding and lysis in both K562 and Molt 4 target cells, but not when the same effectors mediated antibody-dependent cellular cytotoxicity (ADCC). Furthermore, treatment of LGLs with anti-ID antisera for 18 hr enhanced the levels of cytotoxicity. In addition, analyses of supernatants taken from 18 hr anti-ID treatments of LGLs revealed the induction of IFNγ production. Prior studies have shown that IL-2 treatment or pre-incubation of LGLs with K562 cells resulted in the secretion of IFNγ. Our results are consistent with the activation of an NK-R by anti-ID and has lead us to propose that the structure with which the anti-ID reacted is indeed an NK-R.

In addition, recent studies have demonstrated that hetero-cross-linked antibodies, containing one antibody binding site against a receptor on cytotoxic T cells cross-linked with an antibody directed against a target cell surface component, can induce cytotoxic cells to lyse target cells they normally would not destroy. Our results with the cross-linked anti-ID provide strong evidence that the NK-associated molecule is a functional receptor that can trigger cytotoxicity directed by the cross-linking antibody, similar to the T cell receptor (TcR) and the Fc receptor (FcR). Based on SDS-PAGE analysis of immunoprecipitated 125-I labeled CD3- lymphocyte membranes, biochemical studies of this putative receptor yielded a single non-reducible 80-100 and 160 kD proteins. Under reducing conditions, the molecule did not separate into different chains as does the TcR, suggesting that the NK-R is not a heterodimer like the TcR. Flow cytometry studies with unlabeled mouse MAbs [CD2, CD8, CD11, CD16, CD56 indicated that our antiserum was not reacting with a known surface determinant.

Based on the above results, the anti-ID was utilized in screening a human LGL cDNA expression library. A cDNA clone representing the putative NK receptor coding region was isolated. The identity of the clone as the perceived NK receptor was confirmed by the ability of a B-galactosidase partial NK-receptor fusion protein (purified on an anti-B-gal column) to inhibit the immuno-precipitation of both the 80-100 kD and 180 kD proteins. The initial cDNA clone contained a 250 bp insert with an open reading frame coding for 80 amino acids. Neither the nucleotide or amino acid sequence possessed any significant homology to sequences contained in the Genbank sequence database. Southern blot analysis using the cDNA insert demonstrated that this sequence represents a single copy gene in both human and mouse DNA. Analysis of lymphocyte RNA indicated that this gene was found to be expressed as a 6 Kb mRNA in LGLs and a 1.6 Kb mRNA in T cells. The expression and functional importance of the 6 Kb and 1.6 Kb mRNA in NK cells, T cells, both α/β and γ/δ cells and in lymphocytes after various stages of activation, should provide important information regarding the importance of this proposed receptor molecule in the lytic process.

B. Natural Killer Cytotoxic Factor (NKCF).

NKCF is produced as a result of the interaction of murine, rat, or human NK cells and NK-susceptible targets. This factor has been linked to the target cell lysis mediated by the NK effector cell. We exploited the rat NK (RNK) cell lines as an excellent source of NKCF. This provided sufficient NKCF to permit analysis of mouse MAbs (AO287, AO462, and AO316) that significantly inhibit the NKCF cytolytic activity in these LGL-derived supernatants. To further examine the specificity of these antibodies, they were chemically linked to Sepharose-4B and found to absorb a significant proportion of the NKCF cytolytic activity from LGL supernatants while not affecting the TNF reactivities in these preparations. Furthermore, these antibodies demonstrated significant inhibition of cell-mediated cytotoxicity by rat LGLs against YAC-1 target cells. Biochemical analysis of radiolabeled (35-S) RNK-NKCF-containing supernatants indicated that the major protein recognized by these anti-NKCF MAbs had a reduced molecular weight of approximately 12,000 kD by immunoaffinity column purification. The kinetics of RNK-NKCF lysis of YAC-1 cells are identical to NK cell lysis, with maximal lysis seen at 4 hr thus diminishing earlier objections that NKCF could

not be the lytic factor that mediates NK cell lysis due to its slow rate of killing.

Recognizing differences in the ability of RNK-NKCF to lyse YAC-1 and MBL-2 cells, we began studying the mechanism(s) of lysis by RNK-NKCF. Our studies demonstrated that short exposure (<2 hr) to RNK-NKCF demonstrated rapid nuclear degradation potential compared to TNF. This effect is enhanced as the pretreatment time is increased from 0.5 to 2 hrs.

Although the mechanism of lysis by NKCF is not known, results indicate that rapid (<2 hrs) DNA degradation is observed after treatment of YAC-1 cells with NKCF. Because of these results, we have begun studies to examine target cell metabolic requirements using inhibitors of protein and RNA synthesis. These studies indicate that RNK-NKCF is a unique cytolytic molecule with some antigenic similarities to cytolysin.

PUBLICATIONS

Morgan AC Jr, Schroff RW, Ortaldo JR, Herberman RB. Occult leukocyte and tumor-associated antigens by flow cytometry: a review, J Clin Lab Analys 1988;2:182-6.

Grönberg A, Ng J, Ferm MT, Reynolds CW, Ortaldo JR. Analysis of the mechanism of interferon-induced resistance to NK cell-mediated lysis. In: Ades EW, Lopez C, eds. Natural killer cells. Basel: Karger, 1989;188-93.

Mason L, Giardina SL, Hecht T, Ortaldo JR, Mathieson BJ. LGL-1: a non-polymorphic antigen expressed on a major population of mouse natural killer cells, J Immunol 1988;140:4403-12.

Ortaldo JR. Human natural killer cytotoxic factor (NKCF). Relevance, mode of action, and relationship to other cytotoxic factors. In: Ortaldo JR, Ransom J, eds. Leukolysins and cancer. Clifton: Humana Press Inc, 1988;137-47.

Sakamoto S, Ortaldo JR, Young HA. Analysis of the methylation state of the T cell receptor β chain and γ chain genomic DNA in human large granular lymphocytes and T cells. In: Ades EW, Lopez C, eds. Natural killer cells. Basel: Karger, 1989;22-7.

Reynolds CW, Ortaldo JR. Nature and physiology of natural effector cells. In: Shevach E, Oppenheim JJ, eds. Immunophysiology: the role of cells and cytokines in immunity and inflammation. New York: Oxford University Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09283-05 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Control of Human Interferony Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. A. Young	Head	LEI, NCI
Others:	A. Wilt	Laboratory Technician	LEI, NCI
	V. Ciccarone	IRTA Fellow	LEI, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., NCI-FCRF (M. Beckwith, L. Dorman, C. Moratz).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)
- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have identified specific regions of human interferon gamma genomic DNA which can enhance gene expression in murine cells after linkage to a heterologous gene. One enhancer region of DNA lies in the 5′ non-coding region of the gene, appears to be cell specific and is inducible by phorbol esters. The second enhancer region lies in the first intron, is not tissue specific, is stimulated by PMA in T cells but is inhibited by PMA in murine fibroblasts. This same intronic region may be required for gene induction by a combination of PMA and calcium ionophore in a murine B cell line as deletion of intronic DNA containing this enhancer element abolishes inducible gene expression in this cell line. These results indicate that control of interferon gamma gene expression involves multiple DNA regions and that the role of these enhancer elements in gene expression may depend upon the signal transduction pathway utilized.

PHS 6040 (Rev 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Allen Wilt	Laboratory Technician	CMIS, LEI, NCI
Valentina Ciccarone	IRTA Fellow	CMIS, LEI, NCI

OBJECTIVES

We have chosen interferon-γ (IFN-γ) gene expression as a model system for analysis of the control of gene expression in T cells and large granular lymphocytes (LGL). We have found that T cells and LGL are the only two cell types to produce this protein and that IFN-γ mRNA transcription occurs after stimulation of these cells with agents such as interleukin-2 (IL-2) or lectins (e.g. PHA). Thus, the goals of this project have been to define, at the molecular level, the regions of human IFNγ genomic DNA which are involved in the transcriptional activation and mRNA stability of this gene.

ACCOMPLISHMENTS

Upon introduction of human IFN-γ genomic DNA into a murine T lymphoblastoid cell line. We have observed an increase in cytoplasmic IFN mRNA after IL-2 or PMA treatment which takes place in the presence of cycloheximide, indicating that protein synthesis is not required for mRNA induction by either agent. We have also introduced this human genomic DNA into a murine B cell line, Bcl-1. Interestingly, gene expression can only be efficiently induced in the presence of both PMA and calcium ionophore as neither agent alone increases gene expression. Furthermore, deletion of an intronic region of the genomic DNA abolishes this gene induction, indicating that this intronic DNA region may play a role in the stimulation of gene expression by calcium mobilization. An additional unexpected result was obtained as IFN mRNA was induced by LPS in B cells containing either the parental DNA or the DNA containing the first intron deletion, indicating that the signals for LPS gene induction did not require intronic genomic DNA. Thus the overall biological activity of the transfected human IFN-γ genomic DNA indicates that most, if not all, of the regions of DNA required for regulated expression of the gene are contained in the 8.6kb BamH 1 DNA fragment.

Based on these results, we have dissected the non-coding regions of the human DNA to determine which regions enhance gene transcription in response to extracellular signals. This structural analysis of the gene has indicated that enhancer activity can be detected in the 550 bp 5′ to the TATAA box. This enhancer activity demonstrates increased activity after PMA stimulation in the murine T cell line but appears to be inactive in a murine fibroblast cell line. Additional enhancer activity was also localized to a 250 bp region of the first intron, just upstream of a strong DNAse I hypersensitive site also localized in the first intron. This enhancer activity was increased by PMA in the murine T cell line and was also expressed constitutively in the murine fibroblast cell line. In the fibroblasts however, PMA inhibited enhancer activity. This 250 bp

intronic DNA was also part of the DNA found to be needed for gene expression in the murine B cell line.

We have also introduced the human genomic DNA into the germline of the mouse. One line of transgenic mice was observed to produce human IFN after stimulation of spleen cells with lectins. Further analysis of this line indicated that human IFN was only produced under stimulation conditions which resulted in the production of mouse IFN-γ. These results indicate that, in contrast to the results seen in tissue culture, regulated tissue specific expression of the human IFN occurs in the mouse germline. Thus, developmental processes may play an important role in the control of IFN-γ gene expression.

The current studies presented here concerning the regulation of IFN-γ gene expression offer an opportunity to dissect the role of a specific immuno-regulatory molecule in immune system development. By elucidating how the defined regulatory regions are involved in the induction as well as repression of gene expression, one can better understand the pattern of gene expression in vivo. These studies will permit a molecular dissection of the regulation of IFN-γ gene expression during the development of immune system function.

PUBLICATIONS

Sanders, ME, Makgoba, MW, Sharrow, SQ, Stephany, D, Springer, TA, Young, HA, Shaw, S. Human memory T lymphocytes which express increased levels of three cell adhesion molecules (UCHL1, CDw29 Pgp-1) have enhanced gamma interferon production, J Immunol 1988;140:1401-7.

Young, HA, Birchenall-Sparks, MC, Kovacs, EJ, Dorman, L, Ruscetti, FW. Characterization of interleukin-2 and phorbol myristate acetate augmentation of expression of transfected human interferon gamma genomic DNA, J Interferon Res 1988;8:527-38.

Sakamoto, S, Young, HA. Modification of T cell receptor ß chain: The J2 region but not the J1 region of the T cell receptor ß chain gene is hypomethylated in human B cells and monocytes, Nucl Acids Res 1988;16:2149-63.

Garrity, RR, Seed, JL, Young, HA, Winterstein, D, Colburn, NH. Evidence that mouse promotion sensitivity gene prol is transcribed by RNA polymerase III, Gene 1988;68:63-72.

Takacs, L, Kovacs, EJ, Smith, MR, Young, HA, Durum, SK. Detection of IL-1a and IL-1B gene expression by in situ hybridization, J Immunol 1988;141:3081-95.

Kovacs EJ, Radzioch D, Young HA, Varesio L. Control of LPS induced interleukin-1 tumor necrosis factor mRNAs by inhibitors of second messenger pathways in murine macrophages. In: Powa MC, Oppenheim JJ, Kluger MJ Dinarello CA, eds. Monokines and other non-lymphocytic cytokines. New York: Alan R Liss, Inc., 1988;55-60.

Takacs L, Kovacs EJ, Smith MR, Young HA, Durum SK. Detection of IL-1 gene expression by in situ hybridization histochemistry: Tissue localization of IL-1 mRNA in the normal C57BL/6 mouse. In: Powa MC, Oppenheim JJ, Kluger MJ, Dinarello CA, eds. Monokines and other non-lymphocytic cytokines. New York: Alan R Liss, Inc, 1988;89-93.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. Coordinate enhanced expression of three cell adhesion molecules (LFA-2, CD2, LFA-1) and three other molecules (UCHL1, CDw29 Pgp-1) defines a human T cell subset containing memory cells characterized by enhanced gamma interferon production. In: Dupont B, ed. Immunobiology of HLA. New York: Springer-Verlag, 1989;in press.

Kovacs EJ, Radzioch D, Young HA, Varesio L. Different biochemical pathways involved in the expression of interleukin-1 (IL-1) and tumor necrosis factor (TNF) mRNAs in murine macrophages, J Immunol 1988;141:3101-5.

Tartakovsky B, Finnegan A, Muegge K, Brody DT, Kovacs EJ, Smith MR, Berzofsky JA, Young HA, Durum SK. IL-1 is an autocrine growth factor for T cell clones, J Immunol YEAR;141:3863-7.

Smith MR, Young HA, Kung H-F, Durum SK. Intracellular activities of microinjected cytokines and lymphokines. In: Kaplan JG, ed. The cellular basis of immune modulation. New York: Alan R Liss, 1989;in press.

Sakamoto S, Ortaldo JR, Young HA. Analysis of the methylation state of the T cell receptor beta chain gene in T cells and large granular lymphocytes, J Biol Chem 1989;264:251-5.

Bhat NK, Komschlies KL, Fujiwara S, Fisher RJ, Mathieson BJ, Gregorio TA, Young HA, Kasik JW, Ozato K, Papas TS. Expression of Ets genes in mouse thymocyte subsets and T cells, J Immunol 1989;142:672-8.

Kovacs EJ, Beckner SK, Longo DL, Varesio L, Young HA. Cytokine gene expression during the generation of human lymphokine activated killer cells: early induction of interleukin-1B by interleukin-2, Cancer Res 1989;49:940-4.

Ortaldo JR, Young HA, Varesio L. Modulation of CD3- LGL functions by agonist antagonists of PKC: effects on NK and LAK activity and production of IFN gamma, J Immunol 1989;in press.

Thompson CB, Lindsten T, Ledbetter JA, Kunkel SL, Young HA, Emerson SG, Leiden JM, June CH. Activation of the CD28 pathway enhances the production of lymphokines/cytokines by normal human T cells, Proc Natl Acad Sci USA 1989; 86:1333-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09303-03 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Drug Resistance in Cancer Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: H. A. Young Head LEI, NCI

Others: None

COOPERATING UNITS *(if any)*
Program Resources, Inc., NCI-FCRF (R.R.S. Kantor and S. Giardina); DCT, NCI (K. Cowan and A. Towsen); Regina Cancer Institute, Rome, Italy (P.G. Natali)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The present studies examine the mechanisms by which cancer cells develop resistance to cancer chemotherapeutic drugs and seek to devise methods to overcome this resistance. The phase II drug detoxifying enzyme, glutathione S transferase Pi (GST-π) has been previously reported to be over-expressed in several drug resistant tumor cell lines. Monoclonal antibodies to GST-π have been prepared, characterized and the distribution of GST-π determined in normal and neoplastic tissues. GST-π appears to be an extremely useful immuno-histological marker in cancers of the uterine cervix and may also be a marker for colon cancer.

A multimodal approach to cancer treatment has been investigated by the analysis of the effects of combinations of chemotherapeutic drugs, biologicals and immunotoxins on human tumor cell lines. Human colon and ovarian tumor cell lines resistant to chemotherapeutic drugs have been developed by in vivo passage in nude mice. These cell lines are being utilized to analyze the effects of biological response modifiers on the drug resistance phenotype.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young Head CMIS, LEI, NCI

OBJECTIVES

The objectives of this project are: 1) to evaluate the usefulness of the anionic isoenzyme of glutathione S-transferase (GST-π) as a tumor marker in cervical and colon cancer, 2) to study the regulation of the GST-π gene, and 3) to study mechanisms of tumor cell resistance to chemotherapeutic drugs and develop methods to overcome this resistance using combination of biologicals and anti-cancer drugs.

RESULTS

We have previously described the production and characterization of monoclonal antibodies to human glutathione S-transferase π, an enzyme involved in cellular detoxification. This enzyme may be an important prognostic marker in two types of neoplasia; cervical and colon cancers. Immunohistological studies have shown that although normal cervical epithelium does not express GST-π, all grades of cervical carcinoma express GST-π. Correlation of the levels of GST-π in uterine cervical tissue samples and the presence of human papilloma viruses (6, 11, 16, 18, 31, 33) is also being pursued. In colon cancer about 50% of adenomas, >90% of carcinomas and only the most apical portions of normal colonic epithelium express GST-π. Basic studies designed to examine what agents or events can induce or enhance GST-π at the mRNA and protein level are ongoing.

We have established two models of resistance of human tumors to anti-cancer drugs. A human colon carcinoma cell line, HT-29, and an ovarian tumor, OVCAR 3, were passaged in nude mice and selected with either cytoxan or cytoxan/cisplatin combinations. Serial passage of these tumors in nude mice and drug selection have resulted in selection of a drug-resistant phenotype. Present studies are designed to characterize and examine possible mechanisms which underlay this drug resistance. Additionally, we are using these two model systems to evaluate the effectiveness of combinations of biologicals and chemotherapeutic drugs in overcoming drug resistance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09326-01 LEI

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Induction of Cytokine Gene Expression In Vivo by Flavone Acetic Acid

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. A. Young	Head	LEI, NCI
Others:	K. Mace	IRTA Fellow	LMI, NCI
	D. Reynolds	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*

Experimental Therapeutics Section, LEI (R.H. Wiltrout and H. Futami).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have investigated the mechanism by which flavone-8-acetic acid (FAA) acts to enhance immune responsiveness in murine tumor model systems. Analysis of serum samples after FAA administration indicates that both interferon (IFN) α and tumor necrosis factor (TNF) α can be detected in the serum within 1-3 hours. Furthermore, Northern blot analysis of total spleen mRNA was positive for both IFN α and γ as well as TNF α mRNA within 1-2 hours indicating that FAA rapidly induces cytokine gene expression. These results indicate that FAA is a potent biological response modifier which acts by directly stimulating gene expression of important immunoregulatory proteins.

PHS 6040 (Rev 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Kenneth Mace	IRTA Fellow	CMIS, LMI, NCI
Della Reynolds	Biologist	CMIS, LEI, NCI

OBJECTIVES

Flavone Acetic Acid (FAA) augments natural killer (NK) cell activity in both humans and rodents after in vivo administration and synergizes with interleukin-2 for treatment of murine renal cancer. Flavones as a class of natural compounds can have multiple biological effects when utilized in cancer therapy. Some of these effects are similar to those of established anticancer drugs such as direct cytotoxicity and biological response modification (BRM). One possible mechanism for these effects is through the induction of immunoactive cytokines. We have initiated studies to define the kinetics of cytokine mRNA induction after in vivo administration of FAA. The aim of this investigation was to define the induction, in vivo, of mRNA for IFN or other cytokines following FAA, using Northern blot analysis.

ACCOMPLISHMENTS

A panel of cytokine probes was employed in the initial screening of spleen and liver non-parenchymal cell RNA and the temporal expression of induced genes was examined. Spleen cells from untreated and drug-treated mice were assayed for NK activity or used as a source of RNA for Northern blot analysis. NK activity was augmented at 18 hr. after FAA and this level of activity was maintained at 3 days after treatment. Serum Interferon (IFN) and Tumor Necrosis Factor (TNF) were detectable by bioassay following FAA administration with peak levels occurring by 3 hr. post injection. IFN (Type 1 and 2) mRNA in the spleen was upregulated within 1.5-2.0 h after FAA, while an upregulation of TNF mRNA was detected by 1 hr. after treatment. A dose dependent upregulation by FAA of cytokine mRNA and the corresponding serum protein was also observed. No induction of lymphotoxin (TNF-β) or interleukins 1 or 2 mRNA was observed in the spleen in these studies. In liver non-parenchymal cells, TNF-α mRNA was also detected within 2 hours after FAA treatment, indicating that the liver is another target for FAA functional activity. These results demonstrate that FAA can act as a potent inducer of at least two cytokines in vivo, and suggest that the immunomodulatory and immunotherapeutic effects of FAA may be partially mediated by cytokines.

Our increased understanding of how single agents (such as FAA) or combinations of agents (FAA + IL-2) effect cytokine gene expression could allow us to more effectively employ cytokines in cancer therapy. Eventually a hierarchy of therapeutically important effects could be established which would lead to more efficient use of both chemotherapeutic agents and biological response modifiers.

PUBLICATIONS

Hornung, RL, Young, HA, Urba, WJ, Wiltrout, RH. Immunomodulation of natural killer cell activity by flavone acetic acid: occurrence via induction of interferon α/β, J Natl Cancer Inst 1988;80:1226-31.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09262-07 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antitumor Effects of rIL-2 Stimulated Lymphocytes, NK Cells & Macrophages in Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. H. Wiltrout	Head	LEI, NCI
Others:	A. M. Pilaro	IRTA Fellow	LEI, NCI
	E. Ayroldi	Visiting Fellow	LEI, NCI
	S. H. Goey	Guest Researcher	LEI, NCI
	R. Jansen	Guest Researcher	LEI, NCI
	H. Futami	Nakasone Fellow	LEI, NCI
	P. L. Echeagaray	Chemist	LEI, NCI
	M. E. Gruys	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*
CMIS, BRMP (H. Young); LMI, BRMP (F. Ruscetti, J.J. Oppenheim, and L. Varesio), Develop. Therapeutics Program (M. Boyd and D. Zaharko); OAD, BRMP (D. Longo); Program Resources, Inc., NCI-FCRF (T. Sayers, R. Hornung, and J. Keller).

LAB/BRANCH
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Experimental Therapeutics Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies from our laboratory have shown that administration of adoptively transferred lymphokine activated killer (LAK) cells plus IL-2 synergized with chemotherapy to cure 60-80% of mice bearing established murine renal cancer (Renca). LAK cells were fractionated on Percoll density gradients such that >85% of the total cytolytic activity was enriched in approximately 20-50% of the cells in fraction 2 (F2). These F2 cells exhibit increased levels of gene expression for several cytokines, including interferon γ (IFNγ) and tumor necrosis factor α (TNFα). Subsequent studies have shown that the investigational drug Flavone acetic acid (FAA) also induces an upregulation of gene expression for IFNγ and TNFα, as well as for IFNα, and synergizes with rIL-2 for the treatment of Renca. These results led to further experiments in which IFNα and IFNγ were shown to inhibit the proliferation of Renca in vitro and to synergize with IL-2 for treatment of localized Renca in vivo. Additionally, the FAA + IL-2 therapeutic approach resulted in tumor-specific immunity to rechallenge in mice that were previously cured of their disease. Selected cytokines have also been tested for their ability to protect mice from lethal doses of chemotherapy. Specifically, a single injection of transforming growth factor β1 (TGFß1) was shown to transiently inhibit the proliferation of early hematopoietic stem cells in the bone marrow, and to partially protect mice from the lethal effects cyclophosphamide (Cy). Further studies revealed that multiple doses of interleukin 1 (IL-1) also had some chemoprotective effect. Current studies are focused on determining: 1) the mechanism for the chemoprotective effects of TGFß1 and IL-1, 2) the ability of TGFβ1 and IL-1 to synergize for chemoprotection, and 3) whether this chemoprotection translates into a therapeutic benefit through the administration of higher doses or more frequent cycles of Cy to tumor-bearing rodents.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Anne M. Pilaro	IRTA Fellow	ETS, LEI, NCI
Emira Ayroldi	Visiting Fellow	ETS, LEI, NCI
S. Hoo Goey	Guest Researcher	ETS, LEI, NCI
Robertus Jansen	Guest Researcher	ETS, LEI, NCI
Hitoyasu Futami	Nakasone Fellow	ETS, LEI, NCI
Patricia L. Echeagaray	Chemist	ETS, LEI, NCI
M. Eilene Gruys	Biologist	ETS, LEI, NCI

OBJECTIVES

The overall objective of this project is to develop preclinical strategies by which chemotherapeutic drugs and recombinant cytokines can be used in concert for the treatment of cancer in mice. Additional studies are designed to determine the mechanism(s) by which biological response modifiers (BRM) mediate the regression of tumors in vivo. Specific objectives are as follows:

1. To determine the mechanism by which some flavone compounds and rIL-2 synergize for the treatment of established murine renal cancer (Renca), and the basis for induction of specific antitumor immunity during this process.

2. To determine the mechanism(s) by which adoptively transferred lymphokine-activated killer (LAK) cells plus rIL-2 mediate antitumor effects.

3. To determine the role of selected cytokines (TNFα, IFNγ, TGFβ, and IL-1) in the treatment of various mouse tumors by LAK + IL-2 and flavones + IL-2.

4. To study the ability of TGFß1 and IL-1 to protect mice from the lethal effects of high doses of chemotherapeutic drugs.

RESULTS

There are two major areas of study in progress. First, a major focus of the research is on the development and utilization of combination approaches to cancer treatment in mice whereby chemotherapeutic drugs and cytokines are used in the absence of adoptive immunotherapy. Specific approaches developed include: 1) the use of flavones plus rIL-2 for the treatment of Renca and 2) the ability of TGFß1 and IL-1 to protect mice from the lethal effects of chemotherapeutic drugs. Second, studies have been performed to determine whether cytokines produced by adoptively transferred LAK cells actually mediate at least some of their antitumor effects in vivo.

A variety of flavone compounds have been shown to synergize with rIL-2 for the treatment of murine renal cancer. At least part of this synergy is related to the ability of some flavones to stimulate various components of the immune system. For example, we have found that the investigational agent flavone acetic acid (FAA) can modulate NK activity systemically and synergizes with rIL-2 for antitumor activity, in normal and tumor-bearing mice at doses $\geq$ 125 mg/kg. These effects are strictly dose dependent. Subsequent studies performed in collaboration with Dr. Howard Young (CMIS) revealed that these effects may be at least partially regulated by the ability of FAA to induce immunoactive cytokines. Specifically, administration of FAA upregulates the expression of mRNA for TNFα, IFNα, and IFNγ in a strict dose and time-dependent manner. Further, active proteins for each of these cytokines can be detected in the serum of FAA-treated mice. Because of these results, studies have been performed to determine whether these cytokines participate in the therapeutic effects of FAA + rIL-2. Preliminary results obtained in collaboration with Drs. Thomas Sayers and Ronald Hornung (PRI) suggest that IFNα + IFNγ can have direct antiproliferative effects against Renca in vitro and against localized intraperitoneal Renca in vivo. The contribution of these cytokines to the effects of FAA + rIL-2 on systemic Renca are currently in progress. A role for FAA induced cytokines is supported by the observations that mice cured of Renca by FAA + rIL-2 were specifically immune to rechallenge by Renca, and mice cured of intraperitoneal Renca by IFNα, IFNγ, and rIL-2 were also immune to rechallenge. Studies are in progress to determine the role of various T lymphocyte subsets in the induction of specific antitumor immunity by FAA + rIL-2.

Because patients participating in a BRMP intramural Phase I clinical trial of FAA + rIL-2 are undergoing alkalinization as part of their treatment, we have also initiated studies to determine whether alkalization effects the immunomodulatory and immunotherapeutic effects of FAA + rIL-2. To date, we have noted that alkalinization of mice significantly diminishes the therapeutic efficacy of FAA + rIL-2 for Renca, and dramatically reduces the levels of mRNA expressed for TNFα and IFNγ. Experiments are in progress to determine whether these effects can be overcome by administration of higher or repeated doses of FAA.

The antitumor effects of cytokine combinations have also been studied in vitro and in vivo against the GG2EE myelomonocytic tumor. The GG2EE tumor line was obtained by immortalization of C3H/HeJ bone marrow cells by the J2 recombinant retrovirus which bears the v-myc and v-raf oncogenes in the laboratory of Dr. Luigi Varesio, LMI, BRMP. This cell line offers several advantages for the study of therapy by cytokine combinations. First, the GG2EE expresses both the v-myc and v-raf oncogenes. Therefore, antiproliferative effects of cytokines can be studied in the context of changes in oncogene expression. Second, GG2EE is tumorigenic in vivo in athymic mice and therefore cytokines which exhibit antiproliferative effects in vitro can be studied for efficacy in vivo. Our studies have shown that IFNγ and LPS synergize for growth inhibition of GG2EE in vitro. Interestingly, LPS induces GG2EE to express genes for rIL-1α and ß, TNFα, and TGFß. Synergistic or additive antiproliferative effects are also observed when rIL-1 is used in combination with IFNγ and TNFα, while TGFβ also mediates strong antiproliferative effects against GG2EE in vitro. These results suggest that the growth of this cell line can be inhibited in vitro in an autocrine manner following the proper stimulation. Further studies have shown

that IFNγ, IL-1, and TNF also mediate antitumor effects against this cell line in vivo. Studies are in progress to investigate the antitumor effects of cytokine combinations in vivo. Experiments are also in progress to relate the antiproliferative effects of these cytokines to expression of the v-myc and v-raf oncogenes.

Adoptively transferred LAK cells plus IL-2, alone and in combination with chemotherapeutic drugs, have been shown to mediate antitumor effects against a variety of murine tumors. However, the mechanism(s) by which these effects occur remain unclear. We have found that the LAK effector cells can be obtained in highly enriched form from Fraction 2 (F2) of Percoll density gradients. Interestingly these LAK effector cells exhibit pronounced upregulation of the genes for IFNγ and TNFα, two cytokines thought to contribute to the antitumor activity of flavone acetic acid. These observations, combined with previous observations showing that the enriched LAK effector cells do not localize well into sites of tumor growth, suggest that al least some of the antitumor effects of LAK cells in vivo are mediated by soluble cytokines. Studies are in progress to determine whether antibodies to TNFα and IFNγ block the therapeutic effects of LAK cells in vivo.

Because cytokines have been implicated as contributing to the antitumor effects of flavones, LPS, and adoptively transferred LAK cells, we are also investigating their potential use in combination with chemotherapeutic drugs. The results of these studies suggest that because of their pleiotropic effects, selected cytokines might be useful as chemoprotective agents. Preliminary studies performed in collaboration with Dr. Joost Oppenheim (LMI) have shown that rIL-1α can protect mice from lethal doses of cyclophosphamide (Cy) when IL-1 is administered under appropriate dose and time schedules. Specifically, the ip administration of 10,000 to 50,000 U of rIL-1α per day for several days prior to administration of lethal doses of Cy protects up to 70% of the mice. Studies are in progress to determine whether this approach acts by protecting vital bone marrow stem cells. A complete assessment of stem cell viability is being performed by monitoring formation of colony forming units, and a hematological assessment of alterations in leukocyte subsets is also being performed. Parallel studies performed in collaboration with Drs. Frank Ruscetti (LMI), Dan Longo (BRMP), and Jonathan Keller (PRI) have shown that rTGFß1 has pronounced, but transient, effects on the proliferation of early hematopoietic stem cells in the bone marrow. These results demonstrate that a single injection of 1-5μg TGFß1 inhibits the formation of CFU-GEMM colonies when rTGFß1 is administered 24 hrs before assay. This in vivo effect is no longer detectable when the TGFß1 is administered 48 hrs before colony assessment. Thus, we postulated that TGFß1 might protect critical early stem cells from the toxic effects of chemotherapeutic drugs or irradiation. We have already demonstrated that a single ip administration of TGFβ1 does protect about 60-70% of mice from the lethal effects of Cy. Subsequent studies are in progress to: 1) determine the exact mechanism for these effects and 2) determine whether such an approach translates into a therapeutic benefit whereby higher doses, or more frequent cycles, of chemotherapeutic drugs lead to increased tumor regression.

PUBLICATIONS

Sayers TJ, Wiltrout RH. Regulation and function of natural killer cells. In: Rogers TJ, Gilman S, eds. Immunopathology. Caldwell: The Telford Press, 1989; in press.

Pilaro AM, Sayers TJ, Reynolds CW, Ortaldo JR, Wiltrout RH. Chemotaxis of rat large granular lymphocytes (LGL) involves activation of protein kinase C. In: Kaplan JG, Green DR, Bleackley RC, eds. Progress in Leukocyte Biology: Cellular Basis of Immune Modulation. New York: Alan R. Liss, Inc., 1988;9:337-40.

Hornung RL, Salup RR, Wiltrout RH. Tissue distribution and localization of IL-2-activated killer cells after adoptive transfer in vivo. In: Lotzova E, Herberman RB, eds. The Role of Activated Killer Cells and Cancer. Boca Raton: CRC Press Inc, 1988;in press.

Hornung RL, Wiltrout RH, Salup RR. Cancer treatment using IL-2 and IL-2 activated killer cells in combination with chemotherapeutic drugs. In: Lotzova E, Herberman RB, eds. Role of IL-2 and IL-2-activated killer cells in cancer. Boca Raton: CRC Press, Inc., 1988;in press.

Reynolds CW, Wiltrout RH, eds. Functions of the natural immune system. New York: Plenum Press, 1988;485.

Wiltrout RH, Gruys ME, Urias, PE. Inhibition of organ-associated NK activity by 89Strontium. In: Ades EW, Lopez C, eds. Natural killer cells. Basel: Karger, 1989;55-8.

Blasi E, Varesio L, Wiltrout RH. Tumor formation by a murine macrophage cell line immortalized in vitro by v-raf and v-myc oncogenes, Cancer Immunol Immunother 1988;27:109-13.

Ortaldo JR, Mathieson BJ, Wiltrout RH. Characterization and functions of natural killer cells, Ann Inst Pasteur/Immunol 1988;139:444-50.

Hornung RL, Young HA, Urba WJ, Wiltrout RH. Immunomodulation of natural killer cell activity by flavone acetic acid: occurrence via induction of interferon α/β, J Natl Cancer Inst 1988;80:1226-31.

Hornung RL, Back TC, Zaharko DS, Urba WJ, Longo DL, Wiltrout RH. Augmentation of natural killer (NK) activity, induction of interferon, and development of tumor immunity during the successful treatment of established murine renal cancer using flavone acetic-acid (FAA) and interleukin 2, J Immunol 1988;141:3671-9.

Wiltrout RH, Ortaldo JR. New and alternative approaches to adoptive immunotherapy. Proceedings of the International Symposium on New Horizons in Tumor Immunotherapy. Fukuoka, Japan: Second Fukuoka International Symposium, 1988,in press.

Wiltrout RH, Pilaro AM, Gruys ME, Talmadge JE, Longo DL, Ortaldo JR, Reynolds CW. Augmentation of mouse liver-associated NK activity by biological response modifiers occurs largely via rapid recruitment of LGL from the bone marrow, J Immunol 1989;in press.

Pearson JW, Fitzgerald DJP, Willingham MC, Wiltrout RH, Pastan I, Longo DL. Chemo-immunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice, Cancer Res 1989;in press.

Pearson JW, Sivam G, Manger R, Wiltrout RH, Longo DL, Morgan AC. Enhanced therapeutic efficacy against intraperitoneal human tumor xenografts in athymic mice by chemotherapy in combination with immunotoxins, Cancer Res 1989;in press.

Goey H, Keller JR, Back T, Longo DL, Ruscetti FW, Wiltrout RH. Inhibition of early murine progenitor cell proliferation in vivo following locoregional administration of transforming growth factor ß1, J Immunol 1989;in press.

Wiltrout RH, Varesio L. Macrophage-mediated cytotoxicity and suppression. In: Oppenheim JJ, Shevach E, eds. Textbook of immunophysiology: role of cells and cytokines in immunity and inflammation. New York: Oxford Press, 1989;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09282-05 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Potential Differentiation Capacity of Thymocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. H. Wiltrout	Head	LEI, NCI
Other:	T. Gregorio	Biologist	LEI, NCI
	J. Wine	Bio. Lab. Tech.	LEI, NCI
	L. Takacs	Visiting Fellow	LMI, NCI
	F. W. Ruscetti	Head	LMI, NCI
	H. A. Young	Head	LEI, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc. (K. McConville-Komschlies, N. Bhat); LMO, DCE, NCI (T. Papas); Arthritis and Rheumatism Branch, NIAMSD, (W. C. Gause); Vaccine Research and Development Branch, NIAID, (B. J. Mathieson)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Thymocytes can be separated into 5 subsets defined by their surface expression of CD4, CD8, and CD5. These 5 subsets include: 1) the double negatives (DN; CD4-, CD8-, dull and bright CD5+) which contain the most immature cells, 2) the dull Ly-1 (dLyl, CD4-, CD8-, dull CD5+) a subset of DN, 3) double positives (DP; CD4+, CD8+, CD5+) an intermediate stage of differentiation, 4) CD4+ (CD4+, CD8+, CD5+) a mature subset, and 5) CD8+ (CD4-, CD8+, CD5+) another mature subset. This project focuses on the phenotype, differentiation and regulation of these thymocyte subsets. The phenotype of rat and mouse DN thymocytes has been compared. The majority of both rat and mouse DN cells are proliferating when first isolated. However in remarkable contrast to the mouse, the rat DN thymocytes reveal no interleukin 2 receptor (IL-2R) expression. They are also absent of detectable IL-2 mRNA and cytoplasmic IL-2 activity. Examination of estradiol resistant dLyl thymocytes has shown that the dLyl cells remaining in the thymus after short-term estradiol treatment are depleted of bright IL-2R+, large and blast cells yet are still able to generate all CD4 and/or CD8 thymic subsets in irradiated recipients. We have compared the anatomic localization of donor-derived thymocytes following the transfer of various thymic precursors into irradiated recipients. It was found that donor-derived thymocytes are first seen at day 12, after bone marrow cell transfer and are located in the medulla. In contrast, after dLyl transfer, donor-derived cells are first detected by day 6 and are in a speckled pattern throughout all regions of the thymus. Studies on the expression of ets oncogenes in thymocyte subsets has demonstrated that in the adult thymus, ets-1 and ets-2 mRNA expression is 8- to 10-fold higher in the CD4+ subset than in the other subsets examined. Moreover, both the CD4+ and the CD8+ T cell subsets had lower ets RNA levels than the CD4+ thymocytes. Finally, we have observed that the DNA isolated from either CD4+ or CD8+ thymocytes, the more mature thymic subsets, is less methylated in the TCRß region than DNA isolated from the DN population containing the more immature thymocytes.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Theresa Gregorio	Biologist	ETS, LEI, NCI
John Wine	Bio. Lab. Tech.	LEI, NCI
Laszlo Takacs	Visiting Fellow	LMI, NCI
Francis W. Ruscetti	Head	LS, LMI, NCI
Howard A. Young	Head	CMIS, LEI, NCI

OBJECTIVES

The main objectives of this project are: 1) To study the intrathymic differentiation pathways in mice and rats, and to compare the phenotype and functional potential of various thymic subsets from normal or estradiol treated animals after intravenous (i.v.) or intrathymic (i.t.) routes of cell transfer. 2) To determine the anatomic location(s) of thymocyte progenitors and their progeny as differentiated subsets are generated. 3) To examine the regulation of T cell lineage differentiation.

METHODS EMPLOYED

Cell suspensions of immature and mature thymocytes isolated by various methods have been used as potential sources for i.v. and i.t. transfers. Immature dLyl, DN adult thymocytes were obtained by lysis of the CD8+ cells with anti-Ly-2.2 monoclonal antibody (MAb) plus complement (C) treatment, followed by selective cytotoxic elimination of the remaining mature CD4+ subset with anti-L3T4 and anti-Lyl plus C. This complete procedure yields less than 1% of normal adult thymocytes and extreme care is exercised to avoid contamination with blood and peripheral lymphoid tissue. Mature subsets of either CD8+ or CD4+ cells were obtained by first eliminating the reciprocal population by two cycles of MAb + C treatment then selecting the CD8+ or CD4+ cells by adherence to anti-Ig coated plates. Rat DN thymocytes were prepared by adherent depletion with reagents appropriate for the rat CD4 and CD8 antigens.

Purification of cell subsets and differentiation was examined by cell surface phenotypic markers monitored by flow cytometry analysis (FCA) on the LEI FACScan. MAbs and appropriate fluorescent detection reagents have been obtained through the laboratories that produced them, through the ATCC, or through commercial sources for use in these studies.

Congenic animal , used as recipients for thymocyte subsets were irradiated (700-900 rads) or estradiol-treated (50 μg/day x 3 days). These animals have been monitored from 6-40 days for donor and host phenotype after i.v. or i.t. cell transfers. For localization studies, sections of frozen tissue were incubated sequentially with appropriate MAbs, peroxidase-linked anti-Ig reagents for deposition of peroxidase product.

RESULTS

I. Characterization of Immature Thymocytes

Within the population of mouse thymocytes is a small subset of cells, comprising 3-5% of total cells, that are thought to contain the most immature intrathymic precursor. These cells are dull for Ly-1 (CD5; dLyl) by quantitative immunofluorescence, bear no CD4 or CD8 on their cell surface (i.e. double negative, DN) and many are blast cells. These cells have limited self renewal capacity and are committed to the T cell lineage as demonstrated by: a) their ability to repopulate all subsets of thymocytes and T cells and b) their failure to yield multi-potential colony forming units in the spleen when transferred in vivo to irradiated animals. In addition, approximately 60% of these cells express IL-2 receptor (IL-2R). Finally dLyl cells appear to be similar in phenotype to early (day 13-15) fetal thymocytes.

Rats, like mice, express CD4 and/or CD8 on most thymocytes and T cells. The CD4-,CD8-, DN subset of rat thymocytes was isolated and the expression of other phenotypic markers was more extensively studied. Like the majority of mouse DN thymocytes, the rat DN thymocyte subset expresses low levels of CD5 and many of the cells are large, low density, blast cells. However IL-2R expression cannot be detected on the rat DN subset either by antibodies to the 55 kDa chain or by radiolabelled IL-2 binding or by immunoprecipitation after chemical cross-linking. However, the rat thymus does contain some IL-2R+ cells, which appear to be restricted to the medulla, but our data indicate that the medullary IL-2R+ population is not isolated in the DN population. This medullary localization of IL-2R+ cells is also seen in the human thymus, whereas the mouse thymus has both cortical/subcapsular and medullary IL-2R+ cells. Furthermore, rat DN cells do not contain detectable IL-2 mRNA or cytoplasmic IL-2 activity, thus supporting the conclusion that it is unlikely that IL-2 and IL-2R serve to maintain the proliferation of rat DN thymocyte in vivo.

II. Characterization of Estradiol (E2) Resistant dLyl thymocytes.

We have determined that dLyl thymocytes can repopulate the thymus of estradiol-treated animals in a limited fashion. Results have shown that at least DP thymocytes and CD4+ mature thymocytes can be generated after i.t. transfer of dLyl thymocytes to estradiol-treated congenic animals. Whereas irradiation has non-selective lymphocytic effects, estrogen appears to deplete a selected population of subcapsular thymic cells in the adult animal.

We are currently characterizing the subset of dLyl DN cells that remain after estradiol treatment (E2-resistant dLyl). We have found that E2-resistant dLyl thymocytes are depleted of bright IL-2R+, large and blast cells compared to normal controls. Although, all thymic subsets (defined by CD4 and CD8) are generated from E2-resistant dLy-1 cells, the subcapsular region of the thymus is not repopulated with donor-origin cells at any time examined, suggesting that transit through this region may not be essential for generation of thymic subsets. We are continuing this study by examining the ability of E2-resistant dLyl thymocytes to generate peripheral T cells.

III. Intrathymic Localization of Progeny Generated from Thymic Precursors After Transfer.

Studies to monitor the thymic localization of donor-origin thymocytes with time following i.t. or i.v. transfer of thymocyte or bone marrow subsets into irradiated recipients have been performed using the Ly-5 allelic difference and immunoperoxidase detection of MAb binding. Donor-derived thymic cells generated from injected precursors can be detected from days 6 to 26 for dLyl cells transferred i.t. or i.v. and from days 12 to 40 after i.t. bone marrow cell transfer. Whereas, i.v. bone marrow cell transfer permits the establishment of permanent chimeras. In contrast to an expected subcapsular localization, precursors were first observed in the medulla after bone marrow cell transfers and scattered throughout the (day 6) thymus of dLyl recipients. The subsequent localization patterns that were observed suggested that the dLyl differentiation was shifted at least 6 days earlier than bone marrow cell precursors. Additionally, we have observed that differentiation of CD4+, CD8+ thymocytes in irradiated recipients is 3 days ahead of that for dLyl thymocytes.

IV. Expression of ets-oncogenes in Thymocytes and T cell Subsets.

In collaboration with investigators in Program Resources, Inc. and the LMO, DCE, NCI at the FCRF, we have analyzed different subsets of thymic lymphocytes and T cells for ets-oncogenes which appear to have selective expression in lymphocytes. The ets-1 gene was previously shown to be more highly expressed in the mouse thymus than in other tissues. Because the thymic tissue contains various subsets of cells in different stages of proliferation and maturation, we have examined ets gene expression in fetal thymocytes from different stages of development, in isolated subsets of adult thymocytes, and in peripheral T lymphocytes. Expression of the ets-1 gene was first detected at day 18 in fetal thymocytes, corresponding to the first appearance of CD4+ (CD4+, CD8-) thymocytes, and reaches maximal/plateau levels of expression in the thymus at 1 to 2 days after birth. The ets-2 gene expression is detected at least 1 day earlier, coinciding with the presence of both double-positive (CD4+, CD8+) and double-negative (CD4-, CD8-) blast thymocytes and reaches maximal/plateau levels 1 day before birth. In the adult thymus, ets-1 and ets-2 mRNA expression is 10- to 8-fold higher respectively in the CD4+ subset than in the other subsets examined. Higher levels of p55 ets-1 protein were also shown to exist in the CD4+ subset. Because the CD4+ thymic subset is the pool from which the CD4+ peripheral, helper inducer T cells are derived, the ets gene expression was examined in lymph node T cells. Both the CD4+ and the CD8+ T cell subsets had lower ets RNA levels than the CD4+ thymocytes. These results suggest that ets-2 and more particularly ets-1 gene products play an important role in T cell development and differentiation and are not simply associated with proliferating cells, which are observed at a higher frequency in fetal thymocytes, or dull Ly-1 (low CD5+), and double-negative (CD4-, CD8-) adult thymocytes. We are continuing to examine the function of ets-oncogenes in thymocyte differentiation.

V. Methylation State of the T Cell Receptor ß-Chain Gene in Murine Thymocyte Subsets.

Previous analyses of T-cell receptor ß-chain (TCRß) genomic DNA from subsets of human peripheral blood leukocytes suggested that the TCRß methylation pattern

might reflect distinct differentiation pathways. Using murine thymocyte subsets, we have specifically addressed the question of whether methylation of TCRß DNA is related to the cellular maturity and type of TCRß mRNA expressed in the different subsets. We have observed that the DNA isolated from either CD4+ or CD8+ thymocytes, the more mature thymic subsets, is less methylated in the TCRß region than DNA isolated from the DN population containing the more immature thymocytes. Moreover, this pattern of DNA methylation is directly related to the ratio of 1.3 kb to 1.0 kb TCRß mRNA seen in these different cell types. Although a quantitative difference in the level of TCRß was noted for the two mature subsets, no qualitative difference in the ratio of 1.3 kb to 1.0 kb mRNA was detected. Furthermore, these DNA methylation patterns appear to be lineage-related, because the TCRß region of genomic DNA isolated from mouse macrophages is heavily methylated.

PUBLICATIONS

Takacs L, Ruscetti FW, Kovacs EJ, Rocha B, Brocke S, Diamantstein T, Mathieson BJ. Immature, double negative (CD4-,CD8-) rat thymocytes do not express IL-2 receptors, J Immunol 1988;141:3810-8.

Bhat NK, Komschlies KL, Fujiwara S, Fisher RJ, Mathieson BJ, Gregorio TA, Young HA, Kasik JW, Ozato K, Papas TS. Expression of *ets* genes in mouse thymocyte subsets and T cells, J Immunol 1989;142:672-8.

Sakamoto S, Mathieson BJ, Komschlies KL, Bhat NK, Young HA. The methylation state of the T cell antigen receptor ß-chain gene in subpopulations of mouse thymocytes, Eur J Immunol 1989;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09288-04 LEI

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chemo-Immunotoxin Therapy Against Human Tumors Xenografted in Athymic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. W. Pearson	Microbiologist	LEI, NCI
Others:	R. H. Wiltrout	Head	LEI, NCI
	D. L. Longo	Associate Director	BRMP, NCI
	J. R. Ortaldo	Chief	LEI, NCI
	D. FitzGerald	Senior Investigator	LMB, NCI

COOPERATING UNITS *(if any)*
Program Resources Inc. (T. Sayers); Cetus Corp. (E. Groves); NeoRx Corp. (C. Morgan)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.25	1.00	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Xenograft models of a human colon carcinoma (Ht-29) and an ovarian carcinoma (OVCAR-3) are being utilized to define optimal conditions for in vivo intracavitary administration of ITs alone or following drug therapy. Both produce malignant ascites as well as solid tumor growth throughout the peritoneal cavities of athymic mice. The i.p. administration of 300 mg/kg of cyclophosphamide (Cy) plus 100 mg/kg of a chemo-protective drug (WR-2725) to mice bearing Ht-29, 10 and 17 days post-tumor cell inoculation, significantly increased ($p = 0.002$) the MST from 35 days in the control group to 60 days in the drug treated group. Mice that received either two, four, or seven treatments of 0.5 ug OVB-3(PE) following drug therapy exhibited a further significant increase ($p = 0.002$) in MSTs to 81, 87, and 96 days, respectively. Similarly, mice that received multiple treatments of 0.5 ug NR-Lu-10(PE) exhibited a further significant increase ($p = 0.001$) in MSTs of 89, 97, and 105 days, respectively. Tumor cells harvested from mice that showed growth after chemo-immunotoxin therapy exhibited no evidence of either toxin resistance or loss of antigen from the cell surface. The i.p. administration of 20, 10, or 5 ugs of ATF plus the A chain of ricin resulted in highly significant increases in MST's ($p = 0.001$) against an early HT-29 colon tumor. In vitro investigations revealed that both NR-LU-10(PE) and ATF plus the A chain of ricin were highly cytotoxic against OVCAR-3. On going in vivo studies have shown that both ITs appear to be very effective against this peritoneal cancer. Preliminary in vitro data indicate that IFNα potentiates synergistically the antitumor effects of both ITs. A drug combination of Cy and cis-platin (C-P) is also being evaluated against mice bearing this ovarian tumor.

PHS 6040 (Rev 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John W. Pearson	Microbiologist	ETS, LEI, NCI
Robert H. Wiltrout	Head	ETS, LEI, NCI
Dan L. Longo	Associate Director	BRMP, NCI
John R. Ortaldo	Chief	LEI, NCI
David FitzGerald	Senior Investigator	LMB, NCI

OBJECTIVES

The aim of this project is to evaluate the intracavitary administration of immunotoxins (IT) alone or in conjunction with cytoreductive agents against tumors confined to the peritoneal cavities of athymic mice. The specific objectives of this project are as follows: (1) to develop appropriate models for testing the therapeutic efficacy of ITs in vivo, (2) to define optimal treatment protocols, i.e. dose and regimen to maximize the therapeutic effect of specific ITs against localized tumor growth and (3) to assess the feasibility of combination therapy, i.e. chemotherapy in combination with ITs. The models to be utilized in this project are a human colon, Ht-29, and ovarian (OVCAR) carcinomas xenografted into nude mice.

RESULTS

1. Chemo-immunotoxin therapy against Ht-29

Over the past year, studies have been undertaken to explore the therapeutic efficacy of two immunotoxins. A anti-human ovarian monoclonal antibody linked to Pseudomonas exotoxin (OVB-3(PE)) and a anti-human small cell carcinoma linked to PE (NR-LU-10(PE)) have been administered following cytoreductive therapy against a Ht-29 xenografted intraperitoneally (i.p.) into nude mice. Earlier reported studies had demonstrated that the i.p. administration of either IT in mice with low tumor burden, (three days post-tumor) resulted in a highly significant increase in the life span of tumor bearing mice.

Recent studies have shown that the i.p. administration of 300 mg/kg of cyclophosphamide (Cy) plus 100 mg/kg of the chemopreventive drug, WR-2721, 10 and 17 days post-tumor cell inoculation significantly increased ($p = 0.002$) the median survival time (MST) from 35 days in the control group (range 30 to 40 days) to 60 days in the drug treated group, (52 to 65 days). Furthermore, treatment with Cy plus WR-2721 induced a remission period characterized by a lack of grossly detectable ascites of approximately three weeks. After i.p. administration of three million ascites cells on day 0, mice were treated with 300 mg/kg of Cy plus 100 mg/kg of WR-2721 10 and 17 days after tumor cell inoculation. We observed a significant increase in MST (from 37 days to 60 days) in the drug-treated group when compared to the untreated mice ($p = 0.002$). Groups of mice that received either two (14 and 21 days), four (14, 24, 26, and 28 days), or seven (14, 24, 26,28, 31, 33, and 35 days) treatments of 0.5 ug

OVB-3(PE), at a time of minimal tumor burden, demonstrated further significant increases in MST's ($p = < .002$) to 81, 87, and 96 days, respectively, when compared to the group treated with Cy plus WR-2721 alone (MST = 60 days). Likewise, groups of mice that received either two, four, or seven treatments of 0.5 ug NR-LU-10(PE) following cytoreductive therapy exhibited a further significant ($p = 0.001$) in MST of 89, 97, and 105 days, respectively. Therefore, the use of OVB-3(PE) or NR-LU-10(PE) following cytoreductive chemotherapy significantly prolonged survival time of mice bearing the Ht-29 colon tumor over that observed with chemotherapy or ITs alone. Unfortunately, no animals were cured with the combination of Cy plus WR-2721 plus OVB-3(PE) or NR-LU-10(PE). Subsequent in vitro studies revealed that cells harvested from mice that showed growth after chemo-immunotoxin therapy showed no evidence of either toxin resistance or loss of antigen from the cell surface. Therefore, it is felt that the recurrence of the peritoneal tumor is probably due to the failure to completely eradicate this peritoneal disease following chemo-immunotoxin therapy. Currently, studies are underway to potentiate the therapeutic effect of ITs against Ht-29 by administrating a "cocktail" using different MoAbs which are bound to the same toxin or the same MoAb conjugated to two different toxins. Another approach under investigation is the administration of the IT by both i.p. and i.v. routes of injection as there may be tumors cells in sites that are not accessible via the i.p. route of administration. Recently, the i.p. administration of a mouse anti-human transferrin MoAb (ATF) linked to the A chain of ricin was tested in mice with a low tumor burden. The i.p. administration of 20, 10, or 5 ugs of this IT every other day for a total of five treatments beginning three days post-tumor cell inoculation resulted in highly significant increases in MSTs ($p = 0.001$) to 69, 64, and 61 days, respectively, when compared to the untreated control group (MST = 34 days). This IT is currently also being administered following cytoreductive chemotherapy.

2. In Vivo Antitumor Activity of ITs Against OVCAR-3

Studies have been underway involving a human ovarian carcinoma (OVCAR-3) in athymic mice. Following i.p. injection of four million cells, our investigation revealed a localized disease that presented as both malignant ascites and solid tumors throughout the peritoneal cavities of mice. Animals died within 40 to 50 days after tumor cell inoculation. In vitro investigations revealed that both NR-LU-10(PE) and a anti-human transferrin MoAb linked to the A chain of ricin (ATF + A chain of ricin) were highly cytotoxic against OVCAR-3. Therefore, it was of interest to see what effect these two ITs would have against the ovarian tumor when administered at a time of minimal tumor burden. Mice were inoculated i.p. with four million cells on day 0. Five days post-tumor cell inoculation, mice received either 0.5 or 0.25 ugs i.p. of NR-LU-10(PE) every other day for a total of ten treatments. The untreated group exhibited a MST of 47 days. Interestingly, the group of mice that received the higher dose of the IT are tumor free 61 days post-tumor cell inoculation while 50% of the mice that received 0.25 ug of NR-LU-10(PE) remain alive at this time period. A similar approach utilizing 10 or 2.5 ugs of ATF plus A chain of ricin has revealed that all mice are alive and tumor free during this same time frame. Preliminary in vitro studies have shown that sub-optimal doses of human recombinant interferon α (IFNα) synergistically potentiated the antitumor effects of both ITs. Studies are currently underway to evaluate the combination of IFNα and the two ITs against the ovarian tumor when administered at a time of minimal tumor burden.

Finally, investigations are ongoing to evaluate the drug combination of Cy and Cis-platinum (C-P) against localized OVCAR-3 tumor growth in nude mice. Following the i.p. administration of four million OVCAR-3 cells, mice exhibiting physical signs of the disease, 21 days post-tumor, were treated i.p. weekly for two weeks with 100 mg/kg of Cy and 0.5 mg/kg C-P. The mice are currently under observation for a drug induced remission period, lack of grossly visible ascites, as well as quantitation of tumor burden. Once a drug induced remission period has been defined, IT studies will be initiated to determine their therapeutic efficacy following debulking therapy.

PUBLICATIONS

Pearson JW, FitzGerald D, Willingham MC, Wiltrout RH, Pastan I, Longo DL. Chemo-immunotoxin therapy against a human colon tumor (Ht-29) xenografted into nude mice, Cancer Res 1989;49:3562-7

Pearson JW, Sivan G, Manger R, Wiltrout RH, Morgan AC, Longo DL. Enhanced therapeutic efficacy of an immunotoxin in combination with chemotherapy against an intraperitoneal human tumor xenograft in athymic mice, Cancer Res 1989;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09322-01 LEI

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Mechanisms of Leukocyte Migration Following BRM Treatment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. H. Wiltrout	Head	LEI, NCI
CO-PI:	A. M. Pilaro	IRTA Fellow	LEI, NCI
Others:	M. MacPhee	Visiting Fellow	LEI, NCI
	P. L. Echeagary	Chemist	LEI, NCI
	S. E. Olson	Stay-in-School	LEI, NCI

COOPERATING UNITS (if any): Program Resources, Inc., NCI-FCRF, Frederick, MD (T. J. Sayers)

LAB/BRANCH: Laboratory of Experimental Immunology

SECTION: Experimental Therapeutics Section

INSTITUTE AND LOCATION: NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Treatment of mice with various biologic response modifiers (BRM) results in an infiltration of activated large granular lymphocytes (LGL) into peripheral organs such as the liver and spleen. Similarly, injection of specific cytokines, or combinations of cytokines, can mediate the recruitment of different populations of leukocytes into tissue sites. We are using in vitro assays to determine whether different cytokines or other factors released from BRM-treated cells are responsible for the recruitment of LGL and other leukocytes in vivo. To study this, we have developed an in vitro assay for chemotaxis of LGL, using a modified Boyden chamber technique and collagen-coated, millipore filters. LGL movement is quantitated following morphologic or phenotypic staining. Several stimuli have been shown to induce chemotaxis of LGL, and/or other leukocyte subpopulations. Further studies showed that treatment of liver cell cultures enriched for hepatocytes or Kupffer cells with BRM induced the production and release of soluble factors that were chemotactic for LGL in vitro. We will study the effects of these factors as well as known cytokines to determine how BRM regulate the migration of LGL into the liver in vivo. Using the peritoneal cavity as a model, we have begun to study the mechanisms by which different leukocyte types recognize and respond to a stimulus in vivo. In these studies, we have demonstrated that cytokines such as interferon (IFN) and interleukins can recruit specific populations of lymphoid or myeloid cells. These studies will allow us to determine the role of cytokines in the recruitment of specific populations of leukocytes during an inflammatory response. By using these assays, we hope to define the signals which regulate the localization of leukocytes in vivo, and which may therefore be useful in enhancing the localization of antitumor effector cells in sites of tumor growth.

PHS 6040 (Rev 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Anne M. Pilaro	IRTA Fellow	ETS, LEI, NCI
Martin MacPhee	Visiting Fellow	ETS, LEI, NCI
Patricia L. Echeagary	Chemist	ETS, LEI, NCI
Sara E. Olson	Stay-in-School	ETS, LEI, NCI

OBJECTIVES

The overall objective of this project is to examine the mechanism by which specific leukocyte subsets, in particular LGL, monocytes and neutrophils (PMN) are recruited into tissue sites following the administration of BRM. Our studies will focus on the release of active chemotactic factors from BRM-treated tissues as well as on the effects of defined cytokines on leukocyte emigration both in vitro and in vivo. We will also examine other events involved in the homing of leukocytes in vivo, including the interaction of these cells with capillary endothelium and basal lamina. The specific objectives are outlined below:

1. To establish an in vitro method for measuring mouse LGL chemotaxis in response to various stimuli.

2. To study the ability of BRM-induced cytokines to cause chemotaxis of LGL and other leukocyte subsets.

3. To determine the signals which cause LGL and other leukocyte subsets to accumulate in vivo after cytokine treatment.

4. To study the interaction of leukocytes with endothelial cells and basement membrane.

RESULTS

One of the initial goals of this project was to develop a method to rapidly quantitate the movement of LGL in vitro. Our approach was to use isolated rat peripheral blood LGL, since they can be obtained in large numbers and relatively high purity, to study the factors which induce LGL migration. Using the modified Boyden chamber technique and collagen-coated millipore filters, rat LGL were found to respond to a number of different stimuli, including complement fragments, leukotriene B4 and synthetic lipid analogs related to membrane lipids released during tissue injury. Migrating cells were found to be CD8+ LGL by immunoperoxidase staining of the cells on the filter. We have previously demonstrated that treatment of mice with different BRM leads to an accumulation of LGL with natural killer (NK) activity in peripheral organs. Studies have now been initiated to extend these assays to examine factors which induce chemotaxis of mouse NK cells. Since mouse LGL are difficult to identify morphologically, we will use this methodology to specifically identify migrating NK cells in a mixed

population of cells. We will then use these assays to examine the mechanism by which LGL accumulate in peripheral organs following BRM treatment.

Because NK cells have been demonstrated to accumulate in the liver after treatment with BRM, we examined the release of chemotactic factors from isolated liver cell cultures following treatment with various BRM. Preliminary results in the rat system have demonstrated that treatment with lipopolysaccharide (LPS) or the toxic drug acetaminophen (AA) results in a recruitment and activation of LGL in the liver. Treatment of isolated cultures of hepatocytes with these agents resulted in the release of factor(s) into the supernatant fluids which induced LGL chemotaxis. Likewise, treatment of liver macrophages with LPS, or with high doses of the interferon inducer poly ICLC led to a production of LGL chemotactic activity in the supernatant fractions. None of the BRM tested were chemotactic for LGL by themselves. These studies suggest that BRM act indirectly to recruit cells into peripheral tissue sites, probably by inducing the release of chemotactic factors from cells in those organs. Studies are in progress using mouse liver cells to extend and confirm these observations.

One potential mechanism by which NK cells may be recruited in vivo is by the release specific cytokines from BRM-treated tissues. Using the Boyden chamber assay, we have examined the chemotactic effects of several different cytokines on rat LGL. Incubation of rat LGL with human recombinant (hr) interleukin-2 or partially purified rat interferon (IFN) α/β resulted in a 3-5 fold increase in LGL migration. Neither IFNγ nor interleukin-8 was able to stimulate LGL chemotaxis in any of the doses tested. Future studies are planned to examine the effects of other cytokines, such as tumor necrosis factor (TNF) or interleukin-1 on the migration of LGL in vitro.

To extend these studies to examine the effects of cytokines in the in vivo recruitment of LGL, in collaboration with Dr. Thomas Sayers (PRI) we have injected a number of different cytokines into the peritoneal cavity of mice and monitored the infiltration of various leukocyte subsets over time. In initial studies very low doses of interleukin 1 (IL-1) could promote extensive peritoneal accumulation of PMN. Other cytokines like TNF also induced this migration whereas interleukin 2 (IL-2), IFN, and colony stimulating factors (CSFs) did not at the doses we employed. Subsequently we have investigated the effects of i.p. injection of cytokines on the accumulation of murine NK cells. Numbers of NK cells were assessed using 3 different parameters (a) functional: lysis of YAC tumor targets, (b) phenotypic: utilization of the NK specific monoclonal antibody LGL-1 and FACS analysis, (c) enzymatic measuring levels of the enzyme granzyme A (BLT esterase) in cell extracts. Studies from our laboratory have shown that in uninfected animals the vast majority of granzyme A is associated with natural killer cells and levels of enzyme correlate well with numbers of NK cells. Similar findings were noted using all three parameters to assess NK cell number. In contrast to PMN, NK cells did not accumulate in the peritoneum in response to local injection of either TNF nor IL-1. However IFNγ or IL-2 resulted in increases in levels of peritoneal NK without effects on numbers of PMN. Augmentation of peritoneal NK by IL-2 could be significantly inhibited by neutralizing monoclonal antibodies to IFNγ, suggesting that in situ generation of IFNγ played a role in responses to IL-2. Treatment of peritoneal cells with IFNγ in vitro resulted in neither increases in NK lytic activity nor increases in LGL-1+ cells or BLT esterase activity. All consistent with the fact that NK cells

had entered the peritoneal cavity from the circulation rather than been generated from precursors already present in the peritoneal cavity. Furthermore, prior systemic depletion of NK cells by intravenous injection of anti-asialo GM1 antisera completely abrogated accumulation of NK cells in response to IFNγ further indicating that the peritoneal NK cells had migrated into the peritoneal cavity from the circulation. Further studies are envisaged to examine molecular events involving binding of NK cells to vascular endothelial cells as well as basement membrane components in order to define the important molecules involved in extravasation of lymphocytes into non-lymphoid organs or sites.

PMN and other migratory leukocytes must be able to bind to and penetrate endothelial basement membrane before extravasation can occur. Previous results have indicated binding of matrix laminin by specific receptors on PMN may be an important component in leukocyte infiltration. We have used synthetic polypeptides, which are related to the active ligand site of the laminin molecule, to study the role of this receptor in PMN migration. Using the Boyden chamber technique, we studied the effects of these peptides on PMN migration. The synthetic peptide tyr-ile-gly-ser-arg-NH_2 (YIGSR) was found to inhibit PMN chemotaxis to either complement fragments or the synthetic chemoattractant N-formyl-met-leu-phe in a dose-dependent manner. Other structurally related peptides were found to exhibit inhibitory effects on PMN chemotaxis as well. Additionally, adhesion of PMN and macrophages to laminin-coated tissue culture plates was found to be inhibited by approximately 30% when the cells were pretreated with YIGSR. These studies suggest that activation of cell surface binding structures, such as the laminin receptor, may play an integral role in the regulation of different processes associated with leukocyte homing into a tissue. Further studies are planned to examine the role of the laminin receptor in other leukocyte functions associated with emigration in vivo, such as release of lysosomal proteases or activation of the respiratory burst. Additional studies are planned to study the interactions of specific leukocyte subsets with cultured endothelial cells after cytokine treatment. Overall, these studies will provide information on how cytokines released from BRM-treated cells may regulate the infiltration of specific subpopulations of leukocytes into tissue sites, and increase our understanding of the processes involved in lymphocyte homing in vivo.

PUBLICATIONS

Sayers TJ, Twilley TA, Bull CA, Denn AC, Pilaro AM, Lokesh B. Effect of cytokines on polymorphonuclear neutrophil infiltration in the mouse: Prostaglandin and leukotriene-independent induction of infiltration by interleukin I and tumor necrosis factor, J Immunol 1988;141:1670-7.

Pilaro AM, Sayers TJ, Reynolds CW, Ortaldo JR, Wiltrout RH. Chemotaxis of rat large granular lymphocytes (LGL) involves activation of protein kinase C. In: Kaplan JG, Green DR, Bleackley RC, eds. Progress in Leukocyte Biology, Volume 9: Cellular Basis of Immune Modulation. New York: Alan R. Liss, 1988;337-40.

Pilaro AM, Sayers TJ. Chemotaxis of rat peripheral blood large granular lymphocytes to activators of protein kinase C. In: Ades EW, Lopez C, eds. Natural Killer Cells and Host Defense. Basel: Karger, 1989;84-7.

Wiltrout RH, Pilaro AM, Gruys ME, Talmadge JE, Longo DL, Ortaldo JR, Reynolds CW. Augmentation of mouse liver-associated NK activity by biological response modifiers occurs largely via rapid recruitment of LGL from the bone marrow, J Immunol 1989;in press.

SUMMARY REPORT

LABORATORY OF BIOCHEMICAL PHYSIOLOGY

October 1, 1988 through September 30, 1989

INTRODUCTION

The mission of the Laboratory of Biochemical Physiology is to conduct research on the genetic and biochemical events related to the development and expression of the malignant phenotype. An important aspect of this mission is to understand the regulation of cell proliferation/differentiation and to develop strategies for therapeutic intervention in the malignant process based on these findings. We have been focusing on the studies of transcriptional control of gene expression and biochemical mechanisms of signal transduction pathways.

With respect to AIDS-related research, we have been studying the modulation of CD4 expression, the regulation of human immunodeficiency virus (HIV) gene expression, and the biosynthetic pathways of HIV envelope proteins. These studies are aimed at the evaluation of possible targets for drug development and immune intervention. The main research activities of the LBP staff during the past fiscal year are summarized below.

TRANSCRIPTIONAL CONTROL OF CYTOKINE/CYTOKINE RECEPTOR AND HTLV-1 GENES

Human T-cell leukemia virus type I (HTLV-1) encodes a 40 kD nuclear protein, named tax, that transactivates the transcription from the HTLV-1 and several cytokine/cytokine receptor genes (e.g. IL-2R-α, IL-2, IL-3, IL-4, GM-CSF, TNF-α, and TNF-β). The T-cell specific effects of tax on the IL-2 and IL-2R promoters suggest the possibility of an autocrine or paracrine mechanism of T-cell growth as an early event in HTLV-1 mediated leukemogenesis. The HTLV-1 promoter contains three copies of a 21-bp repeat (tax responsive element or TRE), that is involved in tax-mediated induction. Three nuclear factors (TREB-1, TREB-2, and TREB-3) that bind to the TREs were characterized. The "TGACG" motif found in the center of the 21-bp TRE is also present in cAMP responsive elements (CREs) within the cAMP-inducible promoters and in ATF-binding sites (adenovirus Ela-responsive) within the adenovirus early promoters. Indeed, the nuclear factors that bind to the TREs also bind to the CREs and ATF-binding sites. In the case of IL-2R gene, tax transactivates the IL-2R promoter through the TPA-inducible NFκB-like factor(s). Multiple NFκB binding sites were identified. These results suggest that tax functions through the utilization of protein kinase C-or protein kinase A-mediated signal transduction pathways. Molecular cloning and biochemical characterization of these transcriptional factors (TREBs and NFκB-like factors) are in progress.

STUDIES OF G PROTEINS, PROTEIN KINASE C, AND PHOSPHOLIPASE C INVOLVED IN SIGNAL TRANSDUCTION PATHWAYS

We have been studying the biochemical mechanisms of signal transduction pathways leading to cell proliferation/differentiation. The research projects include: 1. *ras* functions; 2. the role of phospholipase in cellular transformation; and 3. molecular cloning of G proteins, protein kinase C, and phospholipase C.

1. Ras functions: The ras gene family codes for a membrane-associated protein p21 closely related to the G-protein family of the signal transduction pathways. We have been focusing on the functional studies of cellular targets/effectors of ras proteins.

Recently, ras GTPase activating protein (GAP) has been purified and cloned. We investigated the physiological role of GAP using Xenopus oocytes as a model system. Microinjection of purified GAP into oocytes markedly induced a rapid increase in the level of diacylglycerol (DG), similar to that observed with oncogenic T24 ras protein. However, only a slight change in the levels of inositol phosphates and phosphoinositides were detected under the same conditions suggesting that DG was derived from phospholipid(s) other than phosphoinositides. Injection of GAP had no further stimulatory effect on DG formation induced by injected T24 ras protein. In contrast to T24 ras protein, injected GAP did not induce oocyte maturation. These results indicate that increase of DG formation alone is not sufficient for the induction of oocyte maturation, and an additional signal(s) mediated by p21 is required for the maturation process. Furthermore, our studies demonstrate a new biological function of GAP involving phospholipid metabolism. We are currently investigating the biochemical mechanisms of action of GAP-mediated phospholipid turnover. Anti-GAP antibodies and anti-sense oligonucleotides of GAP cDNA were prepared, and these reagents will be very useful to study the involvement of GAP in ras functions.

Previously, we have shown that protein kinase C (PKC) potentiates oncogenic ras-induced oocyte maturation. To understand the role of PKC in ras functions, we have purified PKC substrate, 87 kD protein (pp87), from bovine brain. Peptides of pp87 have been microsequenced, and the antibodies against synthetic peptides of pp87 have been developed. These reagents will be used for the functional studies of pp87 in ras/PKC-mediated growth/differentiation pathways.

Finally, we have been working on the cellular proteins which suppress ras functions. Our preliminary results showed the antagonistic effect of two small molecular weight G proteins (smg or rap, ADP-ribosylation factor or ARF) on ras functions. These studies may provide a clue for cancer treatment directed at ras oncogene.

2. The role of phospholipase C in cellular transformation: Inositol phospholipid-specific phospholipase C (PLC) is central to the signal transduction pathway involving in the generation of two second messenger molecules, inositol triphosphate and diacylglycerol. Two PLC isozymes (PLC-I and PLC-II) have been purified from bovine brain. When PLC-I or PLC-II was microinjected into quiescent NIH 3T3 cells, induction of DNA synthesis and morphologic transformation occurred. Our study has established a bioassay for the intracellular mitogenic activity of PLC. In addition, we have identified neutralizing anti-PLC antibodies. Co-injection of anti-PLC antibody with PLC or ras protein blocks induction of DNA synthesis. In contrast, co-injection of the anti-ras antibody (Y13-259) blocks only ras-induced but not PLC-induced DNA synthesis of quiescent NIH 3T3 cells. These results suggest that PLC action is downstream of ras in the signal transduction pathway.

3. Molecular cloning of G proteins, PKC and PLC: Xenopus oocytes became a convenient system for the biochemical studies of signal transduction. However, the proteins (e.g. G proteins, PKC and PLC) involved in signal transduction pathways have not been purified from Xenopus laevis. To characterize these

proteins in Xenopus laevis, we have isolated Xenopus cDNA clones encoding various G proteins (e.g. Goα, Giα, ras, ARF), two types of PLC, and two types of PKC. Based on the sequence data, we are preparing anti-sense oligonucleotides and antibodies against synthetic peptides. These reagents will be used for the functional studies of G proteins, PKC, and PLC. The availability of the cDNA clones will make it possible to express these proteins in large quantities, and to pursue structure-function analysis by site-directed mutagenesis.

MECHANISMS OF ACTION AND RECEPTORS FOR THE INTERFERONS AND OTHER CYTOKINES

We have been studying the expression of receptors and the mechanisms of action of three types of cytokines: the interferons (IFNs), transforming growth factor-β (TGF-β), and interleukin 6 (IL-6).

Our recent studies on the mechanisms of action of the IFNs have demonstrated greater complexity to the IFN-α/β receptor than was originally described. In covalent crosslinking experiments, we have observed two major 125I-rIFN-αA-receptor complexes that are due to the binding of IFN to two separate proteins. These two IFN binding proteins appear to have differing affinities for IFN-α and can be differentially modulated by treatment with low concentrations of IFN-α or with the protein synthesis inhibitor cycloheximide. In studies on signal transduction mechanisms, we have obtained evidence that a functional protein kinase C is required for induction of 2-5A synthetase by rIFN-αA, but that activation of protein kinase C is not the primary or initial signal transduction mechanism. Our previous studies demonstrated a role for either receptor mobility or internalization of rIFN-αA-receptor complexes in responses to IFN-α, suggesting that multiple signaling pathways may be required for maximal responses.

Our studies on TGF-β have demonstrated that this cytokine has synergistic effects with tumor necrosis factor-α in inducing monocytic differentiation. TGF-β also has synergistic effects with retinoic acid in inhibiting phorbol ester-induced transformation of the JB6 epidermal cell line. In both experimental systems, modulation of TGF-β receptor expression may play a role in the observed synergy.

Our studies on IL-6 have demonstrated that the receptors for this cytokine have different molecular weights on different leukocyte lineages. Current studies are addressing the relationships between the various IL-6 binding proteins and the differing biological activities of IL-6 on cells of different lineages.

INTRACELLULAR ACTIVITIES OF TUMOR NECROSIS FACTOR

Previously, we demonstrated that microinjected cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF), are capable of inducing an intracellular response, whereas IL-1 and IL-2 do not have intracellular effects. Our recent studies on the mechanisms of action of TNF killing have demonstrated the activation of a calcium dependent endonuclease by TNF in TNF-sensitive L929 cells. In contrast, TNF-resistant L929 cells were not killed by injection of TNF, and cell lysates did not contain the TNF-activated endonuclease. These results suggest that one mechanism of TNF-induced cytotoxicity may be the activation of a cellular endonuclease by TNF resulting in DNA fragmentation.

AIDS-RELATED RESEARCH

We are evaluating possible targets for the development of anti-AIDS drugs and immune intervention. The research projects include: 1. modulation of CD4 expression; 2. regulation of HIV gene expression; and 3. biosynthetic pathways of HIV envelope proteins.

1. Modulation of CD4 expression: The CD4 glycoprotein is the receptor for the AIDS virus on both T lymphocytes and monocytes. An understanding of mechanisms that regulate CD4 expression and identification of agents, that decrease the level of CD4, may be of utility in prevention of HIV infection. In our studies, we have demonstrated that treatment of monocytic cell lines and peripheral blood monocytes with low concentrations of interferon-γ (IFN-γ) decreases the expression of CD4. This effect of IFN-γ appears to be restricted to cells of monocytic lineage since IFN-γ had no effect on CD4 expression on T lymphocytes. In contrast with the effect of IFN-γ to decrease CD4 expression on monocytes, a monocytic cell product appears to increase CD4 expression. Since monocytes/macrophages are an important reservoir for HIV in vivo, we are continuing our studies on modulation of CD4 expression on monocytes.

2. Regulation of HIV gene expression: In vitro transcription and DNA-binding gel retardation assays have been devised to study the molecular mechanism of HIV gene regulation in monocytes/macrophages. Preliminary results showed the existence of negative regulatory factor(s) in the restricted state of HIV-infected monocytoid leukemic cell line, THP-1. We are currently trying to define the DNA element(s) involved in the negative regulation of HIV expression. Biochemical characterization and molecular cloning (by Southwestern hybridization technique) of the negative regulatory factor(s) are in progress. A recent report showed the activation of HIV gene expression during monocyte differentiation by induction of NFκB (Nature 339:70,1989). Molecular cloning of NFκB-like factors are also in progress. The availability of the cDNA clones will make it possible to pursue structure function studies of the transcription factors involved in HIV gene expression. Modulation of transcription activity could serve as a potential target for AIDS therapy intervention.

3. Biosynthetic pathways of HIV envelope proteins: Glycosylation and proteolytic processing pathways are involved in the biosynthesis of HIV envelope proteins. We studied the effect of chloroquine, an anti-malarial drug known to affect cellular exocytic pathways in HIV system. With chloroquine treatment of virus-infected cells (HIV-1/H9), there was a size reduction of HIV gp120 and extracellular virions derived from treated T-cells contained very little gp120. Infectivity and reverse transcriptase assays carried out with HIV-1 demonstrated that by chloroquine treatment, virus yield was reduced and noninfectious virions were released. The data suggests that inhibition by chloroquine is most likely due to interference with terminal sialylation in the trans-Golgi network. Thus, chloroquine appears to be a potential anti-AIDS drug by the inhibition of infectious virus production. In addition, the continued production of noninfectious virus could induce immunity against the variant glycoproteins of emerging HIV variants. Studies are in progress to determine the effect of chloroquine on HIV-1 and its relatives, produced by other cell types (e.g. monocytes/macrophages), and to evaluate whether chloroquine (or related weak bases) could be useful, either alone or in combination with other drugs (or BRMs), to attack various stages of HIV life cycles.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09299-02 LBP

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Glycosylation and Proteolytic Processing of Human Immunodeficiency Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	W. Tsai	Microbiologist	LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI, NCI-FCRF (B.Q. Li)

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

We studied the effect of chloroquine in Human Immunodeficiency Virus (HIV-1) system. With chloroquine treatment of virus-infected cells, significant size reduction of the cell- and virus-associated surface glycoproteins, gp120, was observed. Infectivity and reverse transcriptase assays of HIV-1 demonstrated that by chloroquine treatment mainly noninfectious virions were released, and virus yield was also reduced. The data suggests that chloroquine inhibition of infectious virus production is most likely due to interference with terminal sialylation in the trans-Golgi network. Thus, chloroquine appears to be a potential drug for therapy of AIDS by stopping production of infectious viruses. In addition, by continuing to produce noninfectious virus, treatment with the drug could allow the production of immunity against the variant glycoproteins of emerging HIV variants. We will test this drug alone or in combination with other drugs in a long term culture condition and on other cell types. Experiments are in progress to establish a primary monocyte/macrophage culture system for this purpose. This system will provide a primary culture to study effects of HIV infection on the expression and/or suppression of cytokines produced by those cells.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Wen-po Tsai	Microbiologist	LBP, NCI

INTRODUCTION

Retroviruses contain two principle envelope proteins, the surface glycoprotein and the transmembrane protein. These two mature proteins are derived from a single large precursor polyprotein encoded by the env gene. The precursor polyproteins are initially co-translationally glycosylated in the rough endoplasmic reticulum. They are further processed by additional glycosylation and proteolysis and transported to the plasma membrane where the cleaved products are incorporated into budding viruses. Inhibition of glycosylation and proteolytic processing of the env gene encoded precursor polyprotein may stop the full expression of viral infectivity. We are performing biochemical studies of HIV env proteins in order to obtain a better understanding of HIV glycosylation pathways and their relation to the viral infectivity.

MAJOR FINDINGS

We have demonstrated the inhibition of HIV infectivity by treatment with chloroquine (an antimalarial drug) in human T-cell lines. The inhibition of infectious virus production is most likely due to the interference with terminal glycosylation in the trans-Gorgi network.

OBJECTIVES AND FUTURE PLANS

The intracellular glycosylation and proteolytic cleavage of human immunodeficiency virus appears to be potential target events for therapy. Studies are in progress to determine the effect of chloroquine on HIV in other cell types, e.g. monocytes/macrophages. We will also evaluate whether chloroquine and its existing analogs or newly synthesized weak bases could be useful, either alone or in combination with other drugs, to attack various stages of the virus life cycle. Similar studies will be carried out with other chemical and biological agents, such as viral protease inhibitors and cytokines.

PUBLICATIONS

Tsai WP, Oroszlan S. Site-directed cytotoxic antibody against the C-terminal segment of the surface glycoprotein gp90 of avian reticuloendotheliosis virus. Virology 1988;166:608-11.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09300-03 LBP

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Microinjection Studies of Biological Response Modifiers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	S. Durum	Senior Staff Fellow	LMI, NCI
	H. Young	Expert	LMI, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI, NCI-FCRF (M. Smith, Y. Liu, J. Keller)

LAB/BRANCH

Laboratory of Biochemical Physiology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2.0	0.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tumor necrosis factor-α (TNF) was shown to have a direct intracellular role inducing cell death. Microinjection of TNF resulted in rapid death of L929 cells within 2 hours compared to the 20 hours that it took soluble TNF to kill these cells. L929 cell lysates were shown to contain a calcium dependent endonuclease that could be activated by TNF. Lysates of cells treated with TNF for 24-48 hours had increased endonuclease activity. TNF-resistant L929 cells were not killed by injection of TNF and cell lysates did not contain a TNF-activated endonuclease. These results suggest that one mechanism of TNF-induced cytotoxicity may be the activation of a cellular endonuclease by TNF. Inositol phospholipid-specific phospholipase C (PLC) is central to the signal transduction pathway involving in generation of two second messenger molecules, inositol triphosphate and diacyl-glycerol. PLC has been shown to induce quiescent 3T3 cells to synthesize DNA and morphologically transform cells. Neutralizing anti-PLC antibody when co-injected with PLC or ras protein blocks induction of DNA synthesis. Co-injection of PLC or ras protein with the neutralizing anti-ras antibody blocks only ras protein induced but not PLC induced DNA synthesis of resting 3T3 cells suggesting that PLC action is downstream of ras in the signal transduction pathway.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Scott Durum	Senior Staff Fellow	LMI, NCI
Howard Young	Expert	LMI, NCI

INTRODUCTION

Microinjection techniques have been used for the studies of the biological activities of physiological important molecules such as cytokines and enzymes. Direct injection, via glass capillary tube, of purified molecules into living cells is a reasonable and effective way to study the biological activities of purified DNA and proteins on appropriate target cells. The research presented in this report uses microinjection as a tool to study the biological mechanisms of important biological response modifiers (BRMs) such as interferon and tumor necrosis factor (TNF) and phospholipase C (PLC).

OBJECTIVES

The goal of this project is to study the biological activities of cytokines after microinjection into appropriate target cells and to compare the intracellular biological response to the normal biological response observed when ligand binds to its surface receptor. These studies directly examine the effects on target cells if the normal receptor binding and subsequent signal transduction pathways are bypassed by microinjection. Injection allows for the assay of intracellular activities of cytokines.

Inositol phospholipid-specific PLC is an important enzyme in phosphoinositide metabolism. It cleaves phosphatidylionositol 4,5-bisphosphate to generate two second messengers, inositol triphosphate and diacylglycerol. The biological ramifications of microinjection of PLC into various cell lines will be studied.

Neutralizing antibodies have been very useful for functional studies of BRMs. We will continue to screen antibodies against important BRMs, such as PLC and oncogene products, for neutralizing activity in the microinjection assay.

METHODS

Microinjection, tissue culture, protein purification, immunological staining, *in situ* hybridization and enzyme assays.

MAJOR FINDINGS

TNFs are known to be involved in the cell mediated cytolysis of natural killer cells (NK), cytotoxic lymphocytes (CTL), and large granule lymphocytes (LGL) of certain tumor cells. Recombinant TNF injected into stationary phase NIH 3T3, L929 or macrophage cells induces a rapid cytolytic effect (within 2 hours). TNF-resistant large granule (LG) fibroblast cells were not killed by injection of TNF at 2.5 ng/ml. As the TNF concentration was increased to 150 ng/ml the same

degree of killing was obtained as with L929 cells. LG fibroblasts have an intracellular mechanism that protects the cell from the cytotoxicity of TNF. A TNF-resistant L929 cell line was also not killed by injection of TNF.

DNA fragmentation was observed in L929 cells following 30-50 hours of TNF treatment and lysate from these cells were shown to contain activated endonucleases. Cytoplasmic supernatants from L929 cells were shown to contain a calcium-dependent endonuclease that can be activated by TNF. Macrophages and TNF-resistant L929 cells do not express this endonuclease activity. Activation of a cytoplasmic endonuclease by TNF may be one mechanism of action of TNF cytotoxicity on target cells.

Serum depleted NIH 3T3 cells are induced to enter the DNA synthesis phase of the cell cycle by microinjection of phospholipase C. This protein is responsible for the enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate forming inositol-triphosphate and diacylglycerol, which act as second messengers for the growth promoting signal transduction of several polypeptide hormones. Microinjection of PLC at 1 mg/ml is cytotoxic and dissolves the cell membrane, while at 100 μg/ml a 25-fold enhancement of ^{3}H-thymidine incorporation into cellular DNA was observed in injected cells compared to control cells.

A PLC neutralizing monoclonal antibody has been identified that blocks the induction of DNA synthesis in quiescent NIH 3T3 following injection of ras protein and PLC. However, if the neutralizing anti-ras monoclonal antibody, Y13-259, is co-injected with ras protein or PLC, the induction of S-phase from PLC is not blocked but ras protein induction of S-phase is blocked. These results demonstrate that ras protein is upstream of PLC in the phosphoinositide signal transduction pathway and that the signal generated by the oncogenic ras protein goes through phospholipase C to induce proliferation.

FUTURE PLANS

1. To continue microinjecting various cytokines into appropriate target cells in order to study the differences between extracellular and intracellular activities of these factors.
2. To study the mechanism of endonuclease activation induced by TNF in sensitive target cells.
3. To use the microinjection assay for continued screening of antibodies against various BRMs that modulate growth and differentiation of cells.
4. To identify which growth factors induced signals can be blocked by anti-PLC/anti-ras monoclonal antibody and to determine the effects of injecting anti-PLC antibody into various oncogene transformed 3T3 fibroblasts.
5. To investigate the mechanism of Ia expression induced by IFN-γ.

PUBLICATIONS

Smith MR, Young HA, Kung HF, Durum SK. Intracellular activities of microinjected cytokines and lymphokines. In: Kaplan JG, ed. The cellular basis of immune modulation. New York: Alan R. Liss, 1988;261-4.

Smith MR, Ryu SH, Suh PG, Rhee SG, Kung HF. S-phase induction and transformation of quiescent NIH 3T3 cells by microinjection of phospholipase C. Proc Natl Acad Sci USA 1989;86:3659-63.

Faltynek C, Kung HF. The biochemical mechanisms of action of the interferons. BioFactors 1988;1:227-35.

Kock A, Schwarz T, Urbanski A, Peng Z, Vetterlein M, Micksche M, Ansel JC, Kung HF, Luger TA. Expression and release of interleukin 1 by different human melanoma cell lines. J Natl Cancer Inst 1989;157:776-82.

Kung HF, Calvert I, Bekesi E, Khan F, Huang KP, Oroszlan S, Henderson LE, Copeland TD, Sowder RC, Wei C. Phosphorylation of human interleukin-2 (IL-2). Mol Cell Biochem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09301-03 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Biological Function of Human Ras Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	S.G. Rhee	Section Head	IR, NHLBI

COOPERATING UNITS *(if any)*
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2.0	0.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ras proteins, like G proteins, have intrinsic GTPase activity and have been postulated to play a role in growth factor receptor-mediated signal transduction. Studies were carried out to understand the regulatory mechanism of ras GTPase activity and the biochemical pathways regulated by ras proteins.

Ras GTPase activating protein (GAP) has recently been identified. To establish the physiological role of GAP, we investigated the role of GAP in ras-mediated actions in vivo. Our results indicate that microinjection of purified GAP into Xenopus oocytes rapidly increases the level of diacylglycerol (DG), similar to that observed with oncogenic T24 ras protein. In contrast to T24 ras protein, injected GAP did not induce oocyte maturation. These results indicate that increase of DG formation alone is not sufficient for the induction of oocyte maturation, and an additional signal(s) mediated by ras is required for the maturation process. Furthermore, our studies demonstrate a new biological function of GAP involving phospholipid metabolism.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Sue Goo Rhee	Section Head	IR, NHLBI

OBJECTIVES

The objectives of this project are to study the biological role of ras proteins in cell transformation, as well as receptor-mediated signal transduction. We have been focusing on the studies of cellular targets/effectors of ras proteins.

MAJOR FINDINGS

1. One major problem in the studies of oncogene functions is the lack of knowledge about effectors for oncogene products. GAP (ras-GTPase activating protein) has recently been purified and cloned. However, the physiological role of GAP has not been established. We investigated GAP functions using Xenopus oocytes as a model system.

Recent studies from other laboratories show that the level of diacylglycerol (DG), an endogenous activator of protein kinase C, is rapidly increased following the introduction of the oncogenic ras proteins into Xenopus oocytes. To test whether GAP influences phospholipid metabolism, oocytes were injected with GAP purified from bovine brain. Our data showed significant increase in the level of DG following microinjection of GAP. We have demonstrated a new biological activity of GAP involving phospholipid turnover.

To determine the involvement of GAP in ras actions, we have prepared antibodies against synthetic peptides of GAP and anti-sense oligonucleotides of GAP cDNA. Studies are in the process to characterize the antibodies and purify them by affinity chromatography. These reagents will be introduced into Xenopus oocytes or cultured cells to examine whether these reagents block ras-mediated biological responses (e.g. induction of cellular DNA synthesis and oocyte maturation).

2. In earlier work, we have shown that protein kinase C enhances oncogenic ras protein-induced oocyte maturation. To explore the important role of protein kinase C in ras actions, we have purified protein kinase C substrate 87 kD protein (pp87) from bovine brain. Peptides of pp87 have been microsequenced, and the antibodies against synthetic peptides of pp87 have been developed. We will use these reagents to study the biological significance of pp87 in ras/PKC-controlled growth pathway.

FUTURE PLANS

By using the reagents we have already developed, we will continue to study the involvement of GAP in ras function; the role of ras/GAP in phospholipid turnover and the biological functions of pp87 in ras/PKC-mediated growth pathway. In another approach, we will focus on the studies of ras suppressor gene products (e.g. smg or rap, ADP-ribosylation factor or ARF).

PUBLICATIONS

Longo PA, Broido MS, Chen J, Kung HF, Pincus MR. The structure of the amino terminal transforming segment of the p21 protein, Tyr^{4}-Thr^{20} (with Asp^{12}), by two-dimensional NMR. Biochem Biophys Res Commun 1988;157:776-82.

Chen K, Peng Z, Lavu S, Kung HF. Molecular cloning and sequence analysis of two distinct types of Xenopus laevis protein kinase C. Second Messenger and Phosphoprotein Research, in press.

Carucci JE, Carty RP, Chung DL, Randall BM, Chen J, Kung HF, Nishimura S, Brandt-Rauf PW, Pincus MR. Blocking of the nucleotide exchange of the p21 protein with the 2', 3'-dialdehyde of guanosine diphosphate, an active site affinity label. Medical Science Research, in press.

Smith MR, Ryu S, Suh P, Rhee S, Kung HF. Transformation of quiescent NIH 3T3 cells by microinjection of phospholipase C. Proc Natl Acad Sci USA 1989;86:3659-63.

Peng Z, Calvert I, Clark J, Helman L, Kahn R, Kung HF. Molecular cloning, sequence analysis and mRNA expression of human ADP-ribosylation factor (ARF). BioFactors, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09302-03 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
G-Proteins and Signal Transduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	S. Lavu	Senior Staff Fellow	LBP, NCI
	I. Calvert	Chemist	LBP, NCI
	R. Kahn	Senior Staff Fellow	LBC, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI (Z. Peng, T. Kamata)

LAB/BRANCH
Laboratory of Biochemical Physiology

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TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Guanyl nucleotide binding proteins (G-proteins) are critical components of several regulatory pathways involved in signal transduction. Recent evidence suggests the involvement of G-proteins in the regulation of various BRMs and alterations in their function may contribute to the pathogenesis of disease. There are a number of small molecular weight G proteins (~21 kD). Of these G-proteins, the ADP-ribosylation factor (ARF) is required for the efficient cholera toxin catalyzed ADP-ribosylation of Gsα. Its physiological role is not known. To investigate ARF function, tissue distribution, and the number of ARF genes in man, we have isolated ARF cDNA clones from a human basal ganglia cDNA library. Comparison of the sequence of human and bovine cDNAs showed 90% identity at the nucleotide level and 100% identity at the amino acid level, demonstrating the highly conserved nature of the ARF protein. Using human ARF cDNA as the probe, we have detected mRNA (~2.2-2.3 Kb) in a wide variety of human tissue and tumor cell lines.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Sukadev Lavu	Senior Staff Fellow	LBP, NCI
Ida Calvert	Chemist	LBP, NCI
Richard Kahn	Senior Staff Fellow	LBC, NCI

INTRODUCTION

Guanyl nucleotide binding proteins (G-protein) are critical components of several regulatory pathways in animal cells, specifically involved in signal transduction. Recent evidence suggests that G-proteins may play a role in mediating the signal transduction of various BRMs, and that any alteration in their function may contribute to the pathogenesis of disease (Evans et al., Science 325:166, 1987 and Growler et al., Nature 327:229, 1987). G-proteins are a multi-gene family. The product of the ras oncogene is also a G-protein. Mutations of this gene appear to be important in oncogenesis, since they are found at increased frequency in a variety of human malignancies. In addition to ras proteins, there are a number of small molecular weight G proteins whose functions are not known.

OBJECTIVES

These studies are aimed at understanding the roles of small molecular weight G proteins in signal transduction, leading to cell proliferation/differentiation.

METHODOLOGY

1. DNA extraction and restriction enzyme analysis.
2. Southern and Northern blottings.
3. Making cDNA libraries and screening for recombinant clones.
4. Detailed characterization of clones.
5. Nucleotide sequence analysis.

MAJOR FINDINGS

A human cDNA clone encoding the full-length ARF was isolated and sequenced, and found to encode for 181 amino acid residues polypeptide (Mr 20,700). Comparison of this G-protein with other G-proteins has revealed close homology at important functional sites (GTP binding, and amino terminal myristylation sites). In order to obtain large quantities of ARF for function studies, ARF cDNA was expressed in E. coli. Our preliminary results showed the antagonistic effect of ARF on ras functions.

FUTURE PLANS

We will investigate the physiological roles of small molecular weight G proteins (e.g. ARF and rap), and their involvement in ras functions. The biochemical mechanisms of action will be studied.

PUBLICATIONS

Longo PA, Broido MS, Chen J, Kung HF, Pincus MR. The structure of the amino terminal transforming segment of the p21 protein, Tyr^{4}-Thr^{20} (with Asp^{12}), by two-dimensional NMR. Biochem Biophys Res Commun 1988;157:776-82.

Kung HF. Biotechnology and Cancer Research. Proceedings of the International Symposium on Human Tumor Markers. Human Tumor Markers, In: Ting SW, Chen JS, Schwarz MK, eds. Amsterdam: Excerpta Medica 1989;231-43.

Brandt-Rauf P, Pincus M, Carty R, Lubowsky J, Avitable M, Kung HF, Maizel J. Conformational effects of amino acid substitutions at positions 10, 12 and 13 in the p21 protein. J Prot Chem 1989;8:79-86.

Carucci JE, Carty RP, Chung DL, Randall BM, Chen J, Kung HF, Nishimura S, Brandt-Rauf PW, Pincus MR. Blocking of the nucleotide exchange of the p21 protein with the 2', 3'-dialdehyde of guanosine diphosphate, an active site affinity label. Medical Science Research, in press.

Peng Z, Calvert I, Clark J, Helman L, Kahn R, Kung HF. Molecular cloning, sequence analysis and mRNA expression of human ADP-ribosylation factor (ARF). BioFactors, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09312-03 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Action and the Receptors for the Interferons and other Cytokines

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	G. Princler	Chemist	LBP, NCI
	M. Schwabe	Visiting Fellow	LBP, NCI
	F. DeBenedetti	Guest Researcher	LBP, NCI
	F.W. Ruscetti	Senior Investigator	LS, LMI, NCI
	L. Falk	IRTA Fellow	LMI, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI (C. Faltynek, D. Radzioch, G. Gusella)

LAB/BRANCH
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INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In studies on the mechanism of action of the interferons (IFNs), we have identified two IFN-α binding proteins with apparent Mr of 90 and 105 kD. These two proteins, which appear to have differing affinities for rIFN-α, can be differentially down-regulated by treatment with low concentrations of rIFN-α. Our studies on signal transduction mechanisms have demonstrated that a functional protein kinase C is required, however, activation of protein kinase C does not appear to be the primary or initial signal transduction mechanism in this response. We have recently radio-iodinated the hybrid recombinant human IFN (rIFN-αA/D). This reagent will be useful for future studies on murine IFN receptor. In studies on transforming growth factor-β (TGF-β), we have demonstrated synergy between TGF-β and tumor necrosis factor-α in inducing monocytic differentiation of human leukemic cell lines. We also observed that TGF-β synergizes with retinoic acid in inhibiting PMA-induced transformation of the JB6 epidermal cell line. Modulation of TGF-β receptor expression may play a role in both cases of observed synergy. In studies on the receptors for interleukin-6 (IL-6), we have shown that the molecular weights of the IL-6-receptor cross-linked complexes vary among different leukocyte lineages.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Gerald Princler	Chemist	LBP, NCI
Michael Schwabe	Visiting Fellow	LBP, NCI
Fabrizio DeBenedetti	Guest Researcher	LBP, NCI
Francis Ruscetti	Senior Investigator	LMI, NCI
Lydia Falk	IRTA Fellow	LMI, NCI
Luigi Varesio	Section Head	IS, LMI, NCI
Nancy Colburn	Chief	CBS, LVC, NCI
Joost Oppenheim	Chief	LMI, NCI

OBJECTIVES

Cytokines are polypeptide hormones that are synthesized in response to specific stimuli and act as intercellular signals to regulate the growth, differentiation, and function of leukocytes and other cell types. We have been studying the biochemical mechanisms of action and the receptors for three types of cytokines: the interferons (IFNs), transforming growth factor-β (TGF-β) and interleukin-6 (IL-6).

The IFNs are a family of proteins with potent antiviral, anti-proliferative and immunomodulatory activities. Although considerable knowledge has been gained in recent years about the biochemical mechanisms of action of the IFNs, there are many aspects that are still poorly understood. Current work emphasizes studies on the structure and modulation of IFN receptors, studies on signal transduction, and studies of the IFNs on the modulation of the expression of specific proteins and mRNAs.

Although TGF-β is a well known regulator of cell proliferation, this cytokine also has other biological activities. In our studies, we have been examining the effect of TGF-β on differentiation of monocytic cells and on phorbol ester-induced transformation. We have also studied the role of TGF-β receptor expression and modulation in these biological effects of TGF-β.

IL-6 has multiple biological activities on various cell types. The objective of our study is to examine the characteristics of the IL-6 receptor on various cell types and to determine whether there is a single type or multiple types of IL-6 receptors mediating the pleiotropic activities of this cytokine.

MAJOR FINDINGS AND FUTURE PLANS

The interaction between IFN-α or -β with its receptor was originally described as the binding to a single class of high affinity receptors. However, more recently, our experiments as well as those of other investigators have demonstrated that this interaction may not be as simple as it was originally envisioned. Biphasic Scatchard plots have been observed, providing evidence for heterogeneity in the binding of IFN-α or -β to its receptor. In covalent cross-linking experiments, one major, rather broad IFN-α-receptor complex was

originally described. However, we have observed that this broad band can be resolved into two complexes with Mr of 115 and 130 kD. These two complexes appear to be due to the binding and cross-linking of IFN-α to two separate proteins, since, using a ligand blotting technique, which is analogous to a Western blot, we observed two IFN-α-binding proteins with Mr of 90 and 105 kD. The higher molecular weight crosslinked complex was preferentially formed in the presence of low concentrations of 125I-rIFN-αA and was preferentially down-regulated after pretreatment with unlabeled rIFN-αA. Moreover, under conditions in which the higher molecular weight complex was preferentially down-regulated, Scatchard analysis of binding data also revealed preferential loss of the higher affinity binding. These results suggest that the 130 kD complex represents the binding and covalent linking of 125I-rIFN-αA to a protein having higher affinity for IFN than the protein which gives rise to the 115 kD crosslinked complex. These two proteins also appear to have differing half-lives in the plasma membrane in the absence of IFN since pretreatment with the protein synthesis inhibitor cycloheximide causes preferential decrease in the subsequent formation of the 130 kD 125I-rIFN-αA-receptor complex. The structural differences between these two binding proteins and the physiological significance of the higher and lower affinity IFN-α receptors which can be differentially modulated remain to be determined.

Many studies on the biological activities of the IFNs have been performed in mice. However, due to the lack of readily available sufficient quantities of recombinant murine IFN-α or -β, previous studies on the murine IFN-α/β receptor have been limited. Although in general human IFNs show little activity on murine cells, a hybrid recombinant human IFN, rIFN-αA/D, is fully active on both murine and human cells. We have recently successfully radioiodinated rIFN-αA/D and demonstrated that 125I-rIFN-αA/D binds with high affinity to the same receptor on murine cells as murine IFN-α and -β. 125I-rIFN-αA/D should prove to be a useful reagent for further studies of murine IFN receptors and the mechanism of action of IFN-α.

The signal transduction mechanisms in the action of IFN-α or-β are not well understood. We have obtained evidence that a functional protein kinase C (PKC) is required for induction of 2-5A synthetase by IFN-α in the Daudi B lymphoblastoid cell line. Specific inhibitors of PKC activity or pretreatment of the cells with phorbol esters to down regulate PKC substantially reduced the ability of IFN-α to induce 2-5A synthetase mRNA and activity. However, several lines of evidence indicate that activation of PKC is not the primary or initial signal transduction mechanism in the induction of 2-5A synthetase. First of all, although PKC inhibitors substantially reduced the induction of 2-5A synthetase mRNA and activity, this effect appeared to be at a post-transcriptional level since these inhibitors did not affect the IFN-induced increase in the rate of 2-5A synthetase transcription. Moreover, although a functional PKC appears to be necessary for induction of 2-5A synthetase mRNA and activity, activation of PKC is not sufficient, since phorbol esters alone or in combination with a calcium ionophore do not mimic the induction of 2-5A synthetase by rIFN-αA. Our previous studies have demonstrated a role for either receptor mobility or internalization of rIFN-αA-receptor complexes in 2-5A synthetase induction, suggesting that multiple pathways may be required for maximal responses to IFN-α.

We have been studying the role for TGF-β in differentiation and proliferation of hematopoietic cells and have found that TGF-β and tumor necrosis factor-α (TNF-α) act synergistically in the monocytic differentiation and growth inhibition of the human leukemic cell lines HL-60 and U937. Moreover, treatment of HL-60 cells with TNF-α increases the expression of TGF-β receptors, suggesting that the synergy between TGF-β and TNF-α may be due, at least in past, to modulation of TGF-β receptor expression.

The JB6 cell line is a nontumorigenic cell line which undergoes transformation when treated with tumor promoters such as PMA. This transformation, which is measured by the ability of the cells to form colonies in soft agar, can be inhibited by a variety of antipromotors such as retinoic acid. In our studies, we have found that TGF-β can also inhibit the tumor promoting activity of PMA and that TGF-β and retinoic acid have synergistic antipromoting activity. Treatment of JB6 cells with PMA was found to decrease the number of high affinity TGF-β receptors whereas retinoic acid prevented this decrease, suggesting a role for TGF-β receptor modulation in the synergistic antipromoting activities of TGF-β and retinoic acid.

To determine whether the diverse biological activities of IL-6 are mediated by the interaction of this cytokine with a single type or multiple types of receptor, affinity cross-linking experiments were performed. In our studies we observed that both the molecular weight and the number of different IL-6-receptor crosslinked complexes varied with cell lineage. Distinct differences in IL-6-receptor crosslinked complexes were found between cells of the monocytic, B lymphocytic and large granular lymphocytic lineages whereas all cell lines examined within a single lineage expressed similar IL-6 receptors. However, analysis of the IL-6 receptor mRNA by Northern blot analysis did not reveal any tissue specific differences in the size of the IL-6 receptor mRNA. The differences in the number of types and sizes of the IL-6-receptor crosslinked complexes may be due to tissue specific post-translational modifications of the IL-6 receptor that may be of biological significance in the action of this cytokine.

PUBLICATIONS

Schwabe M, Princler GL, Faltynek CR. Characterization of the human Type I interferon receptor by ligand blotting. Eur J Immunol 1988;18:2009-14.

Faltynek CR, Kung HF. The biochemical mechanisms of action of the interferons. BioFactors 1988;1:227-35.

Faltynek CR, Schwabe M. Interactions between interferons and other cytokines. Interferons Today and Tomorrow 1988;9:42-4.

Giardina SL, Young HA, Faltynek CR, Jaffe ES, Clark JW, Steis RG, Urba WJ, Mathieson BJ, Gralnick H, Lawrence J, Overton WR, Longo DL. Rearrangement of both immunoglobulin and T-cell receptor genes in a prolymphocytic variant of hairy cell leukemia patient resistant to interferon-α. Blood 1988;72:1708-16.

Faltynek CR, Princler GL, Gusella GL, Varesio L, Radzioch D. A functional protein kinase C is required for induction of 2-5A synthetase by recombinant interferon-aA in Daudi Cells. J Biol Chem, in press.

DeBenedetti F, Falk LA, Ellingsworth LR, Ruscetti FW, Faltynek CR. Synergy between transforming growth factor-β and tumor necrosis factor-α in the induction of monocytic differentiation of human leukemic cell lines. Ann NY Acad Sci, in press.

Oppenheim JJ, Ruscetti FW, Faltynek CR. Interleukins, interferons and other cytokines. In: Stites DP, Stobo JD, Wells JV, eds. Basic and Clinical Immunology Norwalk: Appleton and Lange, 7th edition, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09315-02 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Regulation of HIV Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	F.W. Ruscetti	Microbiologist	LMI, NCI
	C. Samalekos	Visiting Fellow	LBP, NCI

COOPERATING UNITS *(if any)*
PRI, NCI-FCRF (Raziuddin)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

There is accumulating evidence suggesting that the monocyte/macrophage serves as a reservoir for HIV infection and plays a role in the pathogenesis of AIDS. Fresh monocytes can be infected in vitro with HIV. In the brain, the macrophage is the major infected cell type and is associated with the development of neurologic symptoms seen in AIDS patients. In vitro transcription and DNA binding gel retardation assays have been devised to study the molecular mechanism of HIV gene regulation in monocytes/macrophages. Preliminary results showed the existence of negative regulatory factor(s) in the restricted state of HIV-infected monocytoid leukemic cell line, THP-1. We are currently trying to define the DNA element(s) involved in the negative regulation of HIV expression. Biochemical characterization and molecular cloning (by Southwestern hybridization technique) of the negative regulatory factor(s) are in progress. A recent report showed the activation of HIV gene expression during monocyte differentiation by induction of NFκB (Nature, 339,70,1989). Molecular cloning of NFκB-like factors is also in progress. The availability of the cDNA clones will make it possible to pursue structure function studies of the transcription factors involved in HIV gene expression. Modulation of transcription activity could serve as a potential target for AIDS therapy intervention.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Francis W. Ruscetti	Microbiologist	LMI, NCI
Charalambos Samalekos	Visiting Fellow	LBP, NCI

INTRODUCTION

The human immunodeficiency virus (HIV) is the causative agent of AIDS. The infectious viruses show a remarkably varied repertoire of mechanisms for altering or dominating gene expression in host cells. HIV shows a limited cell range and an internally regulated complex pattern of latency and activation, which usually begins with a prolonged latent period without any virus induced cytopathic effect. There is considerable evidence that monocytes and macrophages play a major role in the propagation and pathogenesis of HIV infection. The major cell types infected with HIV in the brain are monocytes and macrophages, leading to the neuropsychiatric abnormalities. The HIV infection, which starts with a prolonged latency, can be overcome by certain biological as well as chemical inducing agents in vitro. At present, the mechanism of release from latency is not clear.

OBJECTIVES

The goal of this project is to investigate the molecular mechanism of HIV gene expression in monocytes/macrophages, which may lead to the development of a sensitive bioassay to quantitate the HIV gene expression in latent as well as restricted state of HIV-infection.

METHODS

1. In vitro transcription assay:

Template-DNA from the recombinant plasmid pL3CAT, (Science 234:988,1986), which contains the LTR promoter enhancer as well as downstream TAR sequences, were gel purified from different restriction sites. Nuclear extracts were prepared according to the standard method (Cell 36:357,1984). The template DNA was preincubated with 15 μl of extracts in the absence of nucleotide triphosphate for 10 minutes at 30°C. The rest of the reaction mixture, 10 μl, containing all the four nucleotide triphosphates, α-^{32}p-UTP, creatine phosphate, salts, and buffers were then added. The typical reaction mixture volume was 25 μl and contained 15 mM HEPES (pH 7.9), 8 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 70 mM KCl, 10% glycerol, 2 mM creatine phosphate, 50 μM ATP, CTP, GTP, 5 μM UTP and 15 μCi of α-^{32}p-UTP. The reaction was continued for 90 minutes at 30°C. The samples were then treated with 50 μg/ml of DNAse I (RNAse free) for 5-10 minutes at 30°C followed by 1 mg/ml proteinase K for 5 minutes at room temperature. The reaction was terminated with 200 μl of stop buffer (7M urea, 100 mM LiCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 100 μg/ml tRNA) and extracted twice with phenol: chloroform: isoamyl alcohol mixture (1:1:0.95), twice with chloroform alone, followed by ethanol precipitation in the presence of 200 μl of 1.0 M

ammonium acetate. The pellets recovered were suspended in 6M urea in 1XTBE and resolved on a denaturing polyacrylamide gel (6% acylamide, 8M urea).

2. DNA-binding gel retardation assay:

The gel retardation assay was a modification of a previously described method (Cell 48:79,1979).

3. Molecular cloning of sequence-specific DNA binding proteins:

Southwestern hybridization technique was used according to the published method (BioTechniques 7:252,1989).

MAJOR FINDINGS

The human monocytoid leukemic cell line, THP-1, was infected with several isolates of HIV. THP-1 cultures were producing HIV 10-17 days after infection. However, 45-60 days post-infection, differences in viral expression were observed among infected culture: 1. latency (provirus with no viral expression); 2. restricted expression (intra-cytoplasmic viral antigens, RNA and virions but no detectable infectious virus released); and 3. continuous production. In restrictedly infected cells, HIV-LTR-directed in vitro transcription showed that nuclei contained material(s) that negatively regulate viral transcription.

FUTURE PLANS

1. Identification of the DNA element(s) involved in the negative regulation of HIV expression.
2. Characterization and molecular cloning of the negative regulatory factor(s).
3. Characterization and molecular cloning of the HIV enhancer-binding proteins.
4. Biochemical characterization of the intra-cytoplasmic viral particles in the restricted state of HIV-infected monocytes.
5. Studies of HIV gene regulation in fresh monocytes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09316-02 LBP

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Signal Transduction in Xenopus Oocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	I. Calvert	Chemist	LBP, NCI
	K. Chen	IRTA Fellow	LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI, NCI-FCRF (Z. Peng, T. Kamata)

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2.0	1.2	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

There is accumulating evidence that phosphoionositol-specific phospholipase C (PI-PLC) is regulated by a G protein in various biological systems. PI-PLC is an important enzyme in phosphoinositide metabolism leading to the generation of two second messengers, inositol triphosphate (IP3) and diacylglycerol (DG). IP3 can release Ca+2 from intracellular stores, and DG is an activator of protein kinase C (PKC). Xenopus oocytes have become an ideal system for the studies of signal transduction. However, these proteins have not been purified from Xenopus laevies. To identify and characterize the proteins involved in signal transduction, we have isolated and characterized cDNA clones encoding various G proteins, two types of phospholipase C, and two species of PKC from Xenopus oocytes cDNA library. Most of these cDNA clones have been fully sequenced. Comparison of the sequences of the coding regions with mammalian cDNAs shows good homology at the nucleotide and deduced amino acid level. The conserved nature of amino acid sequences between Xenopus and mammalian species suggests that the proteins involved in signal transduction are important in evolutionary history. Based on the sequence data, we are preparing anti-sense oligonucleotides and antibodies against synthetic peptides for functional studies of these proteins.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LPB, NCI
Ida Calvert	Chemist	LBP, NCI
Kuang-Hua Chen	IRTA Fellow	LBP, NCI

INTRODUCTION

The G proteins, a family of guanine nucleotide-binding proteins, mediate numerous transmembrane hormonal and sensory transduction processes in eukaryotes. These proteins are essential for coupling the extracellular signals generated by activated membrane receptors to intracellular effector enzymes. There is accumulating evidence that phosphoionositol-specific phospholipase C (PI-PLC) is regulated by a G protein in various biological systems. PI-PLC is an important enzyme on phosphoinositide metabolism leading to the generation of two second messengers, inositol triphosphate (IP3) and diacylglycerol (DG). IP3 can release Ca+2 from intracellular stores and DG is an activator of protein kinase C (PKC). Xenopus oocytes have become an ideal system for the studies of signal transduction. To identify and characterize the proteins involved in signal transduction, we have isolated and characterized cDNA clones encoding Goα, Giα, ADP-ribosylation factor (ARF), ras, two types of phospholipase C, and two species of PKC from Xenopus oocytes cDNA library.

OBJECTIVES

Xenopus oocytes have been used as the model system for the studies of signal transduction pathways. The objectives of this project are to study the structure and function of the proteins involved in signal transduction and the regulation of their expressions.

METHODOLOGY

1. Molecular cloning; 2. DNA sequencing; 3. Southern and Northern blottings; 4. nuclease S1 mapping; and 5. gene expression.

MAJOR FINDINGS

1. Xenopus Goα, Giα, ARF, ras, two types of PI-PLC, and two species of PKC cDNAs have been cloned and sequenced. Comparison of the coding regions with mammalian cDNAs showed good homology suggesting that the proteins are important for their normal function in evolutionary history.
2. Two species of Xenopus PKC cDNAs have been isolated and fully sequenced. The Xenopus PKC sequences share considerable homology with bovine and human PKC-II cDNAs. The sequence within the functional domains of PKC are almost identical among all PKC cDNAs.

FUTURE PLANS

1. To characterize the regulatory regions of Xenopus G proteins, PI-PLC, and PKC genomic clones in order to understand the regulation of expressions of these proteins.
2. To prepare anti-sense oligonucleotides and antibodies against synthetic peptides for functional studies of G proteins, PI-PLC, and PKC.
3. To determine the expression levels of mRNAs at various developmental stages in order to evaluate the possible roles of these proteins in Xenopus development.
4. To express these proteins in large quantities using our expression systems in order to pursue structure function studies.

PUBLICATIONS

Chen K, Peng Z, Lavu S, Kung HF. Molecular cloning and sequence analysis of two distinct types of Xenopus laevis protein kinase C. Second Messenger and Phosphoprotein Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09318-01 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Control of Cytokine/Cytokine Receptor and HTLV-1 Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	J. Zhao	IRTA Fellow	LBP, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI (T. Tan, Y. Huang)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human T-cell leukemia virus type 1 (HTLV-1) encodes tax that transactivates the transcription from the viral (e.g. HTLV-1 and HIV-1) and several cytokine/cytokine receptor genes (e.g. IL-2R , IL-2, IL-3, IL-4, GM-CSF, TNF-α, and TNF-β). The HTLV-1 promoter contains three copies of a 21-bp enhancer element (tax responsive element or TRE), that is involved in tax-mediated induction. Previous results indicate that tax does not interact directly with the DNA, but rather through host-cell proteins. We have characterized three nuclear proteins that bind to the 21-bp TRE, and have demonstrated that the element is also cAMP responsive. Intriguingly, the tax-mediated transactivation can be inhibited by several protein kinase C inhibitors. We have also identified a κB-like element within the IL-2R promoter as the tax- (and phorbol ester-) inducible enhancer. We have demonstrated that the affinity-purified 51 kD NFκB protein bound to this element, and that binding of multiple NFκB-like proteins to the κB element was enhanced in the presence of tax. These results suggest an intriguing possibility that tax functions through the utilization of the protein kinase C- and/or protein kinase A-mediated signal transduction pathways. Molecular cloning and biochemical characterization of these transcriptional factors (TREBs and NFκB-family proteins) are in progress.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Jian Zhao	IRTA Fellow	LBP, NCI

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) has been identified as the etiological agent of adult T-cell leukemia (ATL) and certain chronic neurological diseases including the tropical spastic paraparesis. HTLV-1 encodes a nuclear oncogene product (Science 237:1324, 1987), named tax, that acts in trans to increase the rate of transcription from the HTLV-1 promoter and is required for HTLV-1 replication. The trans-acting viral function of tax has been suspected to be associated with HTLV-1 leukemogenesis. The HTLV-1 promoter contains three copies of a 21-bp repeated enhancer element (tax responsive element or TRE), that is involved both in basal level expression and in tax-mediated induction. Previous results indicate that tax does not interact directly with the TREs but rather through host-cell proteins.

Tax has also been shown to activate the transcription from several viral (HIV-1 and SV40), cytokine-, and cytokine-receptor genes, including IL-2 receptor alpha chain (IL-2R-α), IL-2, IL-3, IL-4, GM-CSF, tumor necrosis factor (TNF-α), and lymphotoxin (TNF-β). The T-cell specific effects of tax on the IL-2 and IL-2R promoters suggest the possibility of an autocrine or paracrine mechanism of T-cell growth, as an early event in HTLV-1 mediated leukemogenesis. Recently, a κB-like element within the IL-2R and HIV-1 promoters as the tax- (and phorbol ester-) inducible enhancer and multiple distinct κB-binding factors (NFκB, H2TF1, EBP-1, HIVEN86A) have been identified. The κB-like enhancer element plays an important role in mediating the induction of HIV-1 gene expression by mitogens (e.g. TPA and cAMP), cytokines (e.g. IL-1 and TNF-α), and viral trans-activators including HTLV-1 tax during monocyte differentiation. Interestingly, a κB-like element within the IL-2 promoter (sequence "GGGATTTCAC"), has been identified as a T-cell specific enhancer element. In addition to the κB-like element, multiple protein binding sites were identified within the IL-2 and IL-2R promoters. Further studies of the above two families of enhancer-binding proteins (TRE-binding proteins and NFκB-family proteins) and other DNA-binding proteins should lead to an understanding of the mechanisms controlling human T-lymphotropic virus (HTLV-1) and T-cell specific IL-2/IL-2R gene expression.

OBJECTIVES

The goal of this project is to investigate the transcriptional control of cellular (cytokines and cytokine receptors) and viral (HTLV-1) gene expression during cell differentiation/proliferation and during infection/transformation by human retroviruses (HTLV-1). The results of this study should provide fundamental information regarding eukaryotic gene expression and host-virus interactions that lead to the development of HTLV-1 associated leukemia/diseases. Recently, various cytokines have been used for the therapeutic treatments of the cancer patients. The information derived from this study should be useful in the future development of treatments and preventions for cancer.

METHODOLOGY

1. Nuclear extracts preparation and in vitro transcription assays.
2. Gel retardation assays and DNase I footprinting analysis.
3. Biochemical characterization and purification of DNA-binding proteins using both conventional and oligonucleotide affinity chromatography.
4. Protein renaturation after SDS-PAGE.
5. DNA transfection and CAT assays.
6. Recombinant DNA techniques.
7. cDNA library screening and DNA sequencing.

MAJOR FINDINGS

We have identified and purified three nuclear proteins that bind to the 21-bp TRE (core sequence "TGACGTCT") within the HTLV-1 LTR, and have demonstrated that the 21-bp TRE is also cAMP responsive. Intriguingly, the tax-mediated trans-activation can be inhibited by several protein kinase C inhibitors. We have also identified a κB-like element within the IL-2R promoter (sequence "GGGAATCTCC") as the tax- (and phorbol ester-) inducible enhancer. We have demonstrated that the affinity-purified 51 kD NFκB protein bound to this element and that binding of multiple NFκB-like proteins to the κB element was enhanced in the presence of tax. The tax responsive (also phorbol ester responsive) κB-like element of the IL-2R promoter does not share any apparent sequence homology with the HTLV-1 21-bp TRE (also cAMP responsive). The fact that tax can trans-activate the viral and cellular genes through either phorbol ester- (e.g. TPA) or cAMP-inducible enhancer element and that protein kinase inhibitors have inhibitory effect upon the tax action suggests an intriguing possibility that tax functions through the utilization of the protein kinase C- and/or protein kinase A-mediated signal transduction pathways. Further studies of the action of these factors from different sources (e.g. latently or lytically infected cells, or cells treated with various inducing agents) should lead to an understanding of (1) the mechanism of action of tax and its role in leukemogenesis and pathogenesis of HTLV-1, (2) the signal transduction mechanisms, and (3) the mechanisms controlling cytokine and cytokine receptor gene expression.

FUTURE PLANS

1. To clone the cDNAs/genes that encode the enhancer-binding proteins of the HTLV-1 and IL-2/IL-2R promoters. The approach will be using the oligonucleotides containing the transcription factor recognition sequences as the DNA probes to screen various T-cell cDNA libraries.

2. These cDNAs/genes, once isolated, will be employed for the in vitro and in vivo (using animal cell culture and transgenic mice systems) structural and functional analysis of individual factors. The proteins produced from these cDNAs using various expression systems should provide a rich resource of these factors for future biochemical studies.

3. To study the HTLV-1 gene regulation (through the 21-bp enhancer element) during the latent to lytic transition/activation.

4. To study the control of lymphokine (IL-2) and lymphokine receptor (IL-2R) gene expression (e.g. through the inducible T-cell specific enhancer elements) during hematopoietic cell proliferation/differentiation and during HTLV-1 infection.

5. As a longer range goal, to clone the T-cell specific transcription factors that regulate the T-cell specific genes (e.g. IL-2 and T cell antigen receptors), and to study the mechanisms controlling tissue (T cell) specific gene expression.

PUBLICATIONS

Ruben S, Poteat H, Tan TH, Kawakami K, Roeder R, Haseltine W, Rosen C. Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-I tax gene product. Science 1988;241:89-92.

Tan TH, Horikoshi M, Roeder RG. Purification and characterization of multiple nuclear factors which bind to the tax-inducible enhancer within the human T-cell leukemia virus type I long terminal repeat. Mol Cell Biol 1989;9:1733-45.

Tan TH, Jia R, Roeder RG. Utilization of signal transduction pathway by the HTLV-I transcriptional activator tax. J Virol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09327-01 LBP

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Modulation of CD4 Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Kung	Chief	LBP, NCI
Others:	F. DeBenedetti	Guest Researcher	LBP, NCI
	I. Espinoza	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI (C. Faltynek, G. Gusella); CIS, PRI (L. Finch)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In studies on the regulation and modulation of the expression of CD4, which is the receptor for HIV-1 on both T lymphocytes and monocytes, we have found that treatment of cells of the monocytic lineage, but not the T lymphocytic lineage with recombinant interferon-γ (rIFN-γ), decreases CD4 expression. Treatment of the promonocytic cell line U937 and freshly isolated peripheral blood monocytes with low concentrations of rIFN-γ decreases the levels of both the CD4 mRNA and the cell surface CD4 antigen. In contrast with the effect of rIFN-γ to decrease CD4 expression, a monocytic cell product that has not yet been identified appears to up-regulate CD4 expression. Several in vitro studies have demonstrated that the IFNs can inhibit the replication of HIV. Since monocytes/macrophages appear to be an important reservoir for HIV in vivo, the effect of rIFN-γ to decrease CD4 expression on these cells may have relevance for the treatment of AIDS.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Fabrizio DeBenedetti	Guest Researcher	LBP, NCI
Igor Espinoza	Visiting Fellow	LMI, NCI

OBJECTIVES

The CD4 glycoprotein is the receptor for the AIDS virus HIV-1 on both T lymphocytes and monocytes. The IFNs are well known antiviral agents and have been recently shown to inhibit the replication of HIV in vitro. The IFNs are also known to modulate the expression of many cellular proteins. The objective of this study is to determine whether the IFNs modulate the expression of CD4, as part of their antiviral activity against HIV. We have also studied the effect of other cytokines, differentiation agents and cell culture conditions on CD4 expression on cells of the monocytic lineage.

MAJOR FINDINGS

Treatment of the promonocytic cell line U937 with rIFN-γ decreased by 50% the cell surface expression of CD4, as measured by cytofluorographic analysis. Maximal decrease was obtained with 20 - 200 U/ml rIFN-γ. Higher concentrations of rGM-CSF and to a lesser extent rIFN-αA and rIFN-β also somewhat decreased CD4 expression on U937 cells. The effect of rIFN-γ to decrease cell surface CD4 expression was due to an actual loss of the CD4 molecule from the cell surface rather than masking of a particular epitope, since similar results were obtained when different antibodies (OKT4 and OKT4A) were used to measure CD4 expression. The effect of rIFN-γ to decrease CD4 expression was not due to a general loss of cell surface proteins, since the binding of OKM1 and anti-HLe-1 increased after rIFN-γ treatment. Freshly isolated normal peripheral blood monocytes treated for 48 hours with rIFN-γ bound much less anti-CD4 antibody than the monocytes incubated in the absence of rIFN-γ. Moreover, treatment with rIFN-γ reduced the percentage of peripheral blood monocytes that were positive for CD4. Concentrations of rIFN-γ as low as 2 U/ml decreased CD4 expression on peripheral blood monocytes. In recent experiments to determine the mechanism for the decreased cell surface CD4 expression, we have observed that treatment of both U937 cells and peripheral blood monocytes with rIFN-γ decreased the level of CD4 mRNA to an extent comparable to the decrease in cell surface CD4 expression.

The ability of rIFN-γ to decrease CD4 expression appeared to be restricted to cells of the myelomonocytic lineage. Treatment with rIFN-γ had no effect on CD4 expression on freshly isolated inactivated peripheral blood T lymphocytes, the MOLT4 leukemic T cell line, or PHA-activated T lymphocytes. In the latter experiment, antibodies to IFN-γ were added during activation of the T cells. Although the antibodies completely neutralized the IFN-γ which had been produced and secreted by the activated T cells, the antibodies had no effect on CD4 levels on the activated T cells. The effect of IFN-γ to decrease CD4 expression on cells of the myelomonocytic, but not T lymphocytic lineage, may be due to the effects of IFN-γ on differentiation of myelomonocytic cells. Indeed we found

that other myelomonocytic differentiation agents such as DMSO also decreased CD4 levels on myelomonocytic cell lines.

In contrast with the effect of rIFN-γ and other differentiation agents to decrease CD4 expression, we observed that a component of U937 conditioned medium increased CD4 expression in these cells. Moreover, incubation of freshly isolated peripheral blood monocytes in vitro for 48 hours also increased CD4 expression. These results suggest that cells of the monocytic lineage produce an unidentified factor that increases CD4 expression.

Because monocytes/macrophages are considered to be a major reservoir for HIV in vivo, the effect of rIFN-γ to decrease CD4 expression on cells of the myelomonocytic lineage may have some clinical relevance for the treatment of AIDS. The level of CD4 expression has been shown to correlate both with susceptibility to HIV infection and with sensitivity to syncytium formation and to the cytopathic effects of the virus. Although the decrease in CD4 expression on rIFN-γ treated monocytes may not be sufficient to prevent HIV infection, it is possible that IFN-γ treated monocytes may be less susceptible to syncytium formation and cell lysis.

FUTURE PLANS

1. To study the effect of other cytokines, differentiation agents, and cell culture conditions on CD4 expression on cells of the monocytic lineage.
2. To characterize the monocytic cell product that appears to up-regulate CD4 expression.

PUBLICATIONS

Faltynek CR, Finch LR, Miller P, Overton WR. Treatment with recombinant IFN-γ decreases cell surface CD4 levels on peripheral blood monocytes and on myelomonocytic cell lines. J Immunol 1989;142:500-8.

SUMMARY REPORT

CLINICAL RESEARCH BRANCH

October 1, 1988 through September 30, 1989

INTRODUCTION

The Clinical Research Branch (CRB) is responsible for the investigation of the therapeutic efficacy, toxicity and mechanisms of action of biologicals and biological response modifying agents in patients with cancer. This branch of the Biological Response Modifiers Program was established to facilitate early clinical trials of biological products with potential as anti-cancer drugs. The unit is located near Frederick Memorial Hospital and was initially opened to the public in May 1981. The unit currently consists of a thirteen-bed inpatient unit that includes a four bed monitoring unit and a separate four bed pheresis unit. The monitoring unit was specifically set up to enable careful management of patients receiving toxic treatment regimens such as high-dose interleukin-2 (IL-2) with or without lymphokine activated killer (LAK) cells. In addition, in January 1989 the outpatient clinic was relocated to a newly renovated clinic adjacent to the hospital. This unit will ultimately house not only the outpatient clinic of the CRB, but also a radiotherapy unit and offices of private local oncologists. The CRB occupies half of the building and includes a four-patient blood drawing area, clinical laboratory, nursing station and waiting area and, currently, twelve-patient beds. Adjacent to the patient care area is the office suite that includes office space for branch physicians, secretaries, data management personnel, and administrative staff.

The CRB research efforts concentrate on in-depth Phase I and Phase II trials of biological response modifiers. Optimal immunomodulating doses as well as maximally tolerated doses of these drugs are being investigated for their therapeutic potential. Additional goals of the branch include the integration of biological therapy with combination chemotherapy and improvement of treatment of patients with Hodgkin's disease and non-Hodgkin's lymphomas.

Over the past year the CRB has continued to expand studies utilizing IL-2 at varying doses with or without the addition of ex vivo activated effector cells. As a follow-up study to our initial investigation of LAK cell and IL-2 therapy in patients with cancer, we designed a study utilizing continuous infusion IL-2 both during the "priming" phase of the study and the LAK cell phase. In addition, because of preclinical data suggesting that adriamycin may enhance trafficking of activated lymphocytes to sites of disease and that cyclophosphamide is capable of eliminating suppressor cell function, we administered cyclophosphamide and adriamycin in low, immunomodulating doses prior to the administration of LAK cells. Finally, interferon alfa, an agent that can synergize with IL-2 in the treatment of cancer, was administered following treatment with LAK cells and IL-2. Unfortunately, despite the favorable preclinical data the overall response rate in melanoma was 22% (9 responders among 41 patients treated) and 14% in renal cell carcinoma (3 responders among 22 patients treated), no different than in previous trials with LAK cells and IL-2 alone. Other protocols have been developed; one includes sequential chemotherapy and immunotherapy for patients with malignant

melanoma, another combines LAK cells, IL-2 and monoclonal antibodies capable of mediating ADCC against either melanoma cells or colon carcinoma cells.

We have also continued our study of chronic moderate dose IL-2 administration in patients with renal cell carcinoma and melanoma. Previous studies have demonstrated that endogenous LAK activity can be generated by prolonged treatment with moderate to high doses of IL-2. In our study, IL-2 was given initially in high doses for three consecutive weeks followed by a lower dose maintenance phase. We serially measured LAK activity and natural killer (NK) cell activity in the peripheral blood of these patients and found that IL-2 when administered in this fashion was capable of inducing sustained low-levels of LAK activity in vivo. However, the response rate in this study was disappointingly low leading to the addition of low doses of cyclophosphamide (capable of reducing suppressor cell activity) to patients receiving the same schedule of IL-2 administration. The results are too early to analyze although immunomodulatory effects are similar to those observed in patients treated with IL-2 alone.

Recent investigations in the laboratories of the Biological Response Modifiers Program have shown that a flavone compound, flavone-8-acetic acid (FAA), has significant immunomodulatory effects in animal models and, when given in combination with IL-2, produces substantial response rates and even cures in animal models in which FAA or IL-2 alone at the same doses are inactive. FAA has been shown to induce the production of tumor necrosis factor, interferon alfa, interferon gamma and to markedly increase peripheral blood and organ-associated NK activity. Following these initial laboratory observations, we began a clinical study of FAA in combination with IL-2 in patients with a variety of malignancies. Major endpoints of this study included antitumor effects, toxicity and examination of the immunomodulatory effects of FAA alone and in combination with IL-2. Because FAA is a weak acid and in theory could precipitate in the renal tubules in acidic urine, patients were routinely hydrated and given sodium bicarbonate to alkalinize the urine. A number of patients have been treated on this trial so far and no immunologic effects other than those expected with IL-2 alone have been observed. Subsequent studies in animals showed that high peak serum levels of FAA are important for both tumor responses and lymphokine induction, suggesting that FAA should be given as a short intravenous infusion rather than as a six-hour infusion as is currently being done in our study. In addition, it was shown that the immunomodulatory and antitumor effects of FAA and IL-2 were abrogated when the animals' urine is alkalinized. Thus, our study is being amended to allow for shorter infusion times of FAA and to delete urinary alkalinization.

Finally, over the past year we have continued to explore the immunomodulatory, toxic and antitumor effects of poly-ICLC in combination with IL-2. Poly-ICLC is one of a family of polyribonucleotides originally studied in humans because of the significant immunomodulatory and antitumor effects that it has in animal models. Previous studies have demonstrated that high doses can be toxic and are associated with only minor antitumor effects. However, lower doses appeared to be better at increasing serum interferon levels but were never explored in detail for antitumor efficacy or in combination with other lymphokines. In this study, decreasing doses of poly-ICLC were administered in combination with IL-2 to evaluate the possibility that doses of poly-ICLC below the maximally tolerated dose have greater immunomodulatory and hopefully greater antitumor activity than maximally tolerated doses. So far however, we have been unable to

detect immunologic changes other than those expected with IL-2 alone. Thus at the doses so far examined, neither enhancement of immunomodulatory effects nor antitumor effects have been observed. Further exploration of lower doses of poly-ICLC are required to complete the evaluation of this potent immunomodulating agent.

The CRB has begun several new studies to investigate the usefulness of colony-stimulating factors (CSF) as myelorestorative agents in combination with chemotherapy and as a biological response modifier in combination with other biologics in the treatment of a variety of malignancies. In one study, recombinant human granulocyte macrophage colony stimulating factors (rHuGM-CSF) is being administered with high-dose carboplatin to patients with refractory ovarian cancer. Patients with ovarian cancer resistant to standard cisplatin chemotherapy have shown significant antitumor effects when treated with carboplatin. Unfortunately, patients can rarely tolerate carboplatin for prolonged periods of time because of its significant myelosuppressive effects. For that reason high doses of carboplatin in patients with refractory ovarian cancer are followed by rHuGM-CSF to accelerate marrow recovery so that subsequent cycles of therapy can be administered without delay and without decreases in dose intensity. Only small numbers of patients have been entered on this trial so far and it is not possible to determine whether GM-CSF has decreased the duration of neutropenia. However, because GM-CSF causes fevers it appears that even if it results in a more rapid recovery of granulocyte counts in these patients, patients will frequently require hospitalization during periods of neutropenia for the administration of empiric antibiotic therapy. GM-CSF also does little for the major toxicity of carboplatin, namely thrombocytopenia. The half-life of transfused random donor platelets in patients receiving GM-CSF may be shortened and several patients have shown refractoriness to random donor platelets during GM-CSF therapy. Some of these patients do not have anti-HLA antibodies in their serum and thus the shortened half-life of transfused platelets appears not to be due to antiplatelet antibodies. Clearly, more needs to be done to determine if GM-CSF will play a role in patients receiving high dose carboplatin therapy. It appears however that the effects of high dose carboplatin on the platelet count have not been ameliorated by GM-CSF and thus CSFs capable of stimulating a more primitive stem cell might allow safer administration of these doses of carboplatin. In this regard, we are conducting a Phase I study of IL-1 alfa to determine its hematopoietic, immunomodulatory and toxic effects in patients with a variety of cancers. This agent has demonstrated some antitumor effectiveness in a variety of preclinical models but also stimulates the proliferation of a more primitive hematopoietic stem cell than does either G- or GM-CSF. The trial with IL-1 is too preliminary to determine if the hematopoietic effects of this agent appear promising enough to warrant investigation with high-dose chemotherapy regimens.

Another trial combining CSFs with intensive chemotherapy regimens has been initiated. In this study, MOPP chemotherapy is being administered to patients with advanced stage Hodgkin's disease in doses higher than with traditional MOPP. This "dose-intense MOPP" is being administered in three-week cycles (instead of the four-week cycle with standard MOPP) and doses of the myelosuppressive agents in this combination have been increased. So far, only three patients have been treated on this regimen and it is too early to determine if GM-CSF can ameliorate the myelosuppressive effects of this regimen. Again, however, because GM-CSF causes fevers, hospitalization of patients during periods of neutropenia and fever has been necessary.

In the past several months, we began a study in which GM-CSF is administered intraperitoneally (i.p.) to patients with tumors limited to the peritoneal space. Animal studies have shown that IP administration of GM-CSF recruits large numbers of monocytes and granulocytes into the peritoneal cavity. Doses capable of increasing peritoneal monocyte and macrophage counts may be substantially lower than doses required to increase peripheral blood monocyte counts. Thus, relatively low doses of GM-CSF might be capable of recruiting effector cells to the site of disease in the peritoneal cavity with only minimal toxic side effects. Monocytes have been shown to specifically recognize and kill tumor cells in vitro and agents known to enhance monocyte cytotoxicity in vivo can bring about tumor responses in laboratory animals. If we are successful at recruiting monocytes to the peritoneal cavity with GM-CSF with acceptable toxicity, we intend to then administer either interferon gamma or IL-2 in conjunction with the GM-CSF to both recruit effector cells and activate them directly at the site of disease. So far, two patients have been treated on this study and substantial increases in monocyte numbers in the peritoneal cavity have been demonstrated even at the starting dose chosen for this trial. Tumor responses have not been observed, however, but these patients have not been treated with GM-CSF in combination with other monocyte activating agents. No unusual toxicities have been observed so far following administration of GM-CSF in this novel way.

In the past year, the CRB has purchased a gamma camera to image patients receiving radiolabeled monoclonal antibodies or radiolabeled effector cells. In the coming years, we intend to significantly increase our investigations of monoclonal antibodies and antibody conjugates in patients with cancer. In the past year, we have participated in a trial of one such immunoconjugate, OVB3-PE, in patients with ovarian cancer. This immunoconjugate consists of a monoclonal antibody that recognizes a cell surface determinant on ovarian carcinoma cells, linked to pseudomonas exotoxin, a potent inhibitor of protein synthesis. We administered this agent i.p. in a Phase I dose escalation study to three patients and observed a minor tumor regression in one retroperitoneal lymph node. At high doses, the immunoconjugate resulted in dose-limiting neurologic toxicity and lower doses are now being more fully explored for antitumor effects. Unfortunately, following intraperitoneal administration, this immunoconjugate rapidly elicited a potent antibody response that was detected as early as two weeks after the first administration of drug. Significant anti-OVB3-PE titers were detected in both the serum and peritoneal fluid. Resistance to the immunoconjugate would likely result if patients were to continue to receive this agent. It appears, therefore, that if this agent is to be used to treat ovarian cancer, multiple doses must be administered over a fairly short period of time prior to the development of anti-immunotoxin antibodies. Such a revised schedule of immunotoxin administration has now been generated and we plan to admit patients to this study shortly.

The CRB has also initiated studies with monoclonal antibodies to the CD3 antigen found on T cells. This antibody (anti-CD3) recognizes a structure associated with the T-cell receptor on both normal and malignant T cells. Anti-CD3 is mitogenic for normal T lymphocytes but when added to cultures of proliferating (malignant or benign) T cells bearing the CD3 antigen, cell cycle arrest and subsequent cell death is observed. Although the mechanism of this antiproliferative effect is unknown, a trial has been initiated to evaluate the efficacy of anti-CD3 in patients with CD3 bearing lymphoproliferative disorders. Two patients with mycosis fungoides have so far been treated at the lowest dose

level and no antitumor or toxic effects were observed. This is a dose escalation study and more patients need to be treated to more fully explore the potential of this antibody in the treatment of these types of malignancies.

Anti-CD3 also stimulates CD3-bearing normal T cells to proliferate and to become nonspecifically cytotoxic for a variety of tumor targets. Only low doses of anti-CD3 are capable of inducing nonspecific cytotoxicity in T lymphocytes with higher doses actually being immunosuppressive. The CRB has initiated a Phase I, dose escalation trial of low doses of anti-CD3 in patients with a variety of tumors in an attempt to determine which doses of this antibody stimulate T-cell function and release of lymphokines. Patients have been treated at three of the six planned dose levels of this study, but not all immunologic monitoring has been completed. We have, however observed an unusual toxic effect seen in only small numbers of patients receiving the high doses typically used to prevent or treat renal allograft rejection. This toxicity was aseptic meningitis and was observed in all three patients receiving 100 micrograms of antibody and in two of nine patients receiving 30 micrograms of antibody. In each case, the patients developed fever, photophobia, nuccal rigidity and a lymphocytic CSF pleocytosis. Dose-limiting toxicity was reached at the 100 microgram dose level and further patients are being admitted to the 30 microgram dose level to more fully evaluate immunologic alterations brought about by this antibody. In the coming year we will complete the evaluation of the immunologic effects of this antibody and initiate trials of anti-CD3 stimulated T cells in combination with lymphokines in patients.

Over the past year the CRB has continued its studies of patients with hairy cell leukemia. We continue to administer interferon to a large study population of such patients to determine the long-term effectiveness and safety of this agent. Thirty-four patients of the original 52 responding patients continue on therapy. Six patients have stopped interferon because of the development acquired interferon resistance. All of these patients developed neutralizing anti-interferon antibodies that probably mediated the interferon resistance. Interferon resistance was only seen in those patients with neutralizing interferon antibodies and not in those with either non-neutralizing antibodies or no detectable antibodies. The association of interferon resistance and the presence of neutralizing antibodies is statistically significant. Two patients developed an increase in their red cell mass late in the course of therapy with interferon. Interferon was discontinued in one patient without effect on the hematocrit. This patient subsequently underwent a one unit pheblotomy but two months later died of a small bowel venous thrombosis. Both patients were thoroughly investigated for conditions associated with secondary erythrocytosis and none were found. Thus, erythrocytosis may be a late effect of this therapy and should be aggressively treated with phelobotomy if detected.

Previous studies of interferon in hairy cell leukemia in which therapy was administered for either 12 or 18 consecutive months indicated that complete remissions were very rare and relapse and or disease progression was the rule once interferon was discontinued. Patients will certainly require either prolonged continuous therapy or intermittent therapy to maintain normal peripheral blood counts. The effectiveness and safety of long-term interferon therapy as administered in our study will be compared with the results obtained in other studies to determine if prolonged continuous therapy is the treatment of choice after an initial response to interferon has been obtained.

We have also continued to evaluate the usefulness of deoxycoformycin in patients with hairy cell leukemia refractory to or intolerant of interferon alfa. No new patients have been admitted to this study over the past year because interferon resistance has not developed in any more patients on our interferon study. However, of the three patients who achieved a significant partial response on this study all three continue in partial remission without evidence of disease progression. Clearly, deoxycoformycin has activity in this setting but longer follow-up will be required to determine if additional therapy will be needed.

Because of the effectiveness of deoxycoformycin in the setting of interferon resistance, a study combining sequentially administered interferon alfa and deoxycoformycin was initiated. This study completed accrual last year and all patients have now completed treatment. Response to therapy on this study was judged by serial evaluations of peripheral blood counts and sequential examinations of bilateral iliac crest bone marrow biopsies and aspirates. We observed no complete remissions in this study; all patients showed small numbers of hairy cells in bone marrow biopsies at the completion of therapy. Thus deoxycoformycin and interferon alfa administered in this fashion are not curative. However, with a median follow-up of two years off therapy no patient has shown a progressive increase in the number of hairy cells in the bone marrow. We continue to follow these patients carefully for any evidence of disease progression. The results of this study conflict with those reported by other investigators using single-agent deoxycoformycin. In these studies, complete, long-lasting remissions have been reported but only when single unilateral bone marrow biopsies were examined for evidence of residual disease. These more relaxed criteria for complete remission may be the reason why complete remissions were reported with deoxycoformycin therapy and might explain the differences between our trial and previously published results. We would conclude however that with appropriate marrow evaluations patients with hairy cell leukemia are not rendered disease-free with deoxycoformycin and therefore curative therapy likely does not yet exist for this disease.

A fundamental difference between immunotherapy and chemotherapy for cancer is that biological agents may be most active at doses substantially lower than the maximally tolerated dose. A previous study conducted by the CRB indicated that in patients with malignant melanoma rendered disease-free with surgery but at high risk for recurrence, the maximally tolerated interferon gamma dose was no more effective than a much lower dose in enhancing monocyte hydrogen peroxide generation. The results of this study have been used as the basis for an adjuvant study of interferon gamma in a similar cohort of patients. In the past year, we have initiated a similar trial in patients with measurable metastatic malignant melanoma to determine the optimal immunomodulating dose of interferon gamma in this setting and to determine the antitumor effectiveness of this dose. The initial phase of the study, in which patients are assigned to one of three interferon gamma doses, is now nearing completion. Preliminary laboratory data suggest that the optimal immunomodulating dose will be similar to that determined in the earlier study. The antitumor effectiveness of this optimal immunomodulating dose has not yet been investigated.

Over the past year a number of chemotherapy trials for different types of non-Hodgkin's lymphomas have been opened at the CRB. In addition to studying newer therapeutic approaches to these diseases, the availability of tissue and blood from these patients will allow investigations into the immunologic aberrations and molecular biology of these disorders. The first such study will investigate

the efficacy of combination chemotherapy in patients with angiocentric immunoproliferative lesions. This group of diseases includes disorders traditionally felt to be premalignant or inflammatory processes. It has been suggested that the diseases comprising angiocentric immunoproliferative lesions are in reality a spectrum of diseases ranging from disorders with only a propensity for later evolution into lymphomas to overt angiocentric lymphomas. Previous studies in the National Institutes of Allergy and Infectious Diseases utilized cyclophosphamide and prednisone in patients with so called Grade I or II angiocentric immunoproliferative lesions (previously known as lethal midline granuloma or lymphomatoid granulomatosis). Although most patients responded to such therapy approximately one-half later developed overt lymphoma that was resistant to standard combination chemotherapy regimens. Paradoxically, patients who presented to the National Cancer Institute with angiocentric lymphoma who were treated with aggressive combination chemotherapy had high rates of complete remissions that have proven to be durable. In this study, we will treat patients with all grades of angiocentric immunoproliferative lesions with combination chemotherapy to not only induce remissions of overtly malignant disease but also to attempt to prevent the evolution of "benign" angiocentric immunoproliferative lesions to chemotherapy-resistant lymphoma. All three evaluable patients so far entered on this study have achieved a complete remission but further study is necessary to determine the appropriateness of this approach. These disorders generally represent proliferations of T lymphocytes and the availability of tissue specimens from these patients will hopefully allow in-depth characterization of the disease process.

The CRB has also begun to admit patients to a dose-intense modified version of the original ProMACE-Cytabom chemotherapy regimen in patients with advanced stages of diffuse aggressive lymphomas. In this study ProMACE drugs are administered in weekly alternating fashion with Cytabom drugs for 12 consecutive weeks. In this manner patients are continuously exposed to drug combinations active in lymphoma. This method of administering ProMACE-Cytabom will hopefully result in high rates of durable complete remissions. Too few patients have been entered to assess the long-term efficacy of this approach.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09291-04 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Alternating 2'-dCF and Recombinant Leukocyte A IFN in Hairy Cell Leukemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. G. Steis	Medical Officer	CRB, NCI
Others:	A. Martin	Staff Fellow	IDB, CTEP, NCI
	S. Nerenstone	Staff Fellow	CIB, CTEP, NCI
	D. L. Longo	Associate Director	OAD, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
- ☒ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Because we have previously observed substantial responses to deoxycoformycin in hairy cell leukemia after failure of interferon, we began this pilot protocol to evaluate the efficacy and toxicity of combined treatments with recombinant interferon alfa-2a and 2'deoxycoformycin. Response criteria in this protocol were more strict than others in that we required absence of hairy cells from bilateral iliac crest bone marrow biopsies obtained twice at approximately two month intervals and improvement of the peripheral blood hemoglobin to greater than 12 grams percent, granulocyte counts to greater than 1500 per cubic millimeter, and platelet counts to greater than 100,000 per cubic millimeter. Most previous studies using deoxycoformycin have declared patients complete responders on the basis of a single unilateral bone marrow biopsy. Of 15 patients entered, 14 are evaluable for response with one patient with diffuse osteosclerosis not being evaluable for marrow response. All patients had normalization of peripheral blood counts and all 14 patients evaluable for marrow response had partial responses with only small numbers of residual hairy cells remaining in the bone marrow. Had marrow responses been based on only single unilateral bone marrow biopsies, we would have had 10 complete responses. No patient has had any disease progression in the bone marrow with a median follow-up off treatment of 2 years. Although initial responses to the combination of deoxycoformycin and interferon appeared to be no better than that observed with deoxycoformycin alone, the duration of response may be longer. We continue to follow these patients to determine the response duration.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Allison Martin	Staff Fellow	IDB, CTEP, NCI
Stacy Nerenstone	Staff Fellow	CIB, CTEP, NCI
Dan L. Longo	Associate Director	OAD, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. To determine the qualitative and quantitative toxicities of 2'-deoxycoformycin and interferon alfa given in alternating monthly cycles to patients with progressive hairy cell leukemia.

2. To gather preliminary data on the efficacy of this treatment combination by response rate and remission duration.

3. To determine the alterations in immune function as a result of treatment with alternating deoxycoformycin and alfa interferon.

4. To determine the biochemical consequences of administering deoxycoformycin with a biological response modifier.

METHODS EMPLOYED

Patients to be considered eligible for this study must have morphologically identifiable hairy cells in the peripheral blood, bone marrow or tissue biopsies including a positive stain for tartrate-resistant acid phosphatase. Peripheral blood count criteria include a hemoglobin less than 10 grams percent or patients must be transfusion dependent and/or the platelet count must be less than 100,000 and/or the absolute granulocyte count must be less than 1,500. Patients must have a performance status of greater than 70 percent and have an expected survival greater than three months prior to study entry. Prior therapy is allowed for patients entering this study, but no patients must have received prior alfa interferon or deoxycoformycin. Patients were evaluated in the outpatient clinic of the Clinical Research Branch and were hydrated and given 4 mg/m2 of deoxycoformycin intravenously as a one-half hour infusion. After infusion of the deoxycoformycin, a further liter of fluid was administered to insure adequate hydration. Patients were serially evaluated for myelosuppression and receive weekly injections at this dose of deoxycoformycin for three consecutive weeks. On week 4 of the study no therapy was given, and in weeks 5 through 8 daily subcutaneous injections of recombinant alfa interferon at a dose of 3 million units/m2 is administered. This two-month cycle of deoxycoformycin alternating with interferon was repeated for a minimum of 7 cycles. Initial response was evaluated by serial evaluations of peripheral blood counts and by bilateral iliac crest bone marrow biopsies performed at the 6-month time point. Bilateral iliac crest bone marrow biopsies were then obtained every two months thereafter and every six months after the completion

of therapy. For patients clearly responding well to therapy and tolerating the therapy reasonably well, some of the deoxycoformycin injections were administered by the local physician. These local physicians, however, must agree to follow the protocol exactly as written.

MAJOR FINDINGS

Fifteen patients were admitted to this trial, and 14 patients are now evaluable for response. Again, one patient is not evaluable for response because of diffuse bony sclerosis making it impossible to obtain evaluable marrow biopsies. All evaluable patients had a partial response to therapy with a reduction in the number of hairy cells in the bone marrow to less than 5 percent of the total cellularity and improvement in peripheral blood counts to the levels mentioned previously. Had the usual criteria for a complete response been used in our study, 10 patients would have had a complete remission. However, with our requirement for bilateral bone marrow biopsies done twice a minimum of two months apart, no patient was a complete responder. The duration of response after completion of therapy remains to be determined. With a median follow-up of 2 years from the end of therapy, none of our patients have had progression of marrow disease and none have had worsening blood counts or the development of splenomegaly.

The toxicity of this therapy consisted of nausea and vomiting in thirteen patients, depression in eight, and paresthesias in five, dermatitis in ten, minor eye irritation without corresponding clinical signs in six and herpes zoster infections in four. The herpes zoster infections occurred late and may be indicative of an underlying suppression of immune function. Hematologic toxicity included progressive neutropenia in all 15 treated patients early in the course of therapy, anemia requiring transfusions in three patients, and fever requiring admission to the hospital for antibiotics in five patients. Ten patients had less than 500 granulocytes/mm3 early in the course of therapy, but most of these patients had granulocyte counts less than 500 prior to beginning deoxycoformycin.

Because deoxycoformycin is an inhibitor of adenosine deaminase and deficiency of this enzyme is associated with some types of congenital immunodeficiencies, we evaluated immune function in these patients serially and compared their immune function to patients receiving only interferon alfa. Patients receiving deoxycoformycin had rapid and very marked drops in total lymphocyte counts and in total numbers of CD4 and CD8 positive lymphocytes. Although most patients had normal CD4 positive cell levels and normal T4 to T8 ratios prior to beginning therapy, all patients had decreases in CD4 positive lymphocyte numbers to less than 200/mm3 shortly after the first course of deoxycoformycin therapy. CD4 positive cell numbers remained depressed for the duration of therapy and remained depressed for up to six months after completion of deoxycoformycin. The duration of suppression of CD4 positive cell counts is being followed closely in these patients. As mentioned above, four patients had herpes zoster infections late in the course of therapy or after completion of therapy on this study. Three were treated with acyclovir early in the course of therapy, and it is impossible to know whether they would have had disseminated disease had this therapy not been given. The fourth patient was not treated with Acyclovir and

only had localized dermatomal disease. No patients in our study have had opportunistic infections to date.

SIGNIFICANCE

This study was undertaken primarily to improve the long-term response duration and complete remission rates of patients with hairy cell leukemia. Although initial studies with deoxycoformycin suggested that this drug was capable of inducing a high rate of complete remission, and that these complete remissions were durable, subsequent studies have shown that relapses are occurring with increasing frequency. Because our definition of complete remission is different from that used in other studies, it is impossible to compare response rates directly. However, it does not appear that the combination of alfa interferon and deoxycoformycin will lead to substantially greater degrees of initial response than expected with deoxycoformycin alone. However, only by comparison of the duration of response in our patients to the duration of response in patients receiving deoxycoformycin alone (and graded for response with more relaxed criteria) will it be possible for us to determine if this therapy is better, the same, or not as good as deoxycoformycin alone. Clearly, this regimen is reasonably well tolerated and brings about peripheral blood and marrow responses much more rapidly than does interferon alfa.

An additional point of interest in this study is that when one looks carefully in the bone marrow for residual disease, it can uniformly be found. Thus, other studies in hairy cell leukemia patients in which deoxycoformycin is being used need to now be re-evaluated by more extensive testing of the bone marrow as was done in our study to determine if true complete responses had occurred or if the apparent high complete remission rate in previous studies was merely related to sampling error of the bone marrow.

In addition, the profound immunosuppression in our patients and its very long duration has not previously been documented in patients receiving deoxycoformycin. This aspect of this therapy will have to be evaluated closely in our study and should also be done in other studies in which deoxycoformycin is being used. Prolonged follow-up of these patients is necessary to determine the late clinical consequences of this immune suppression. In trying to decide between deoxycoformycin or interferon treatment for hairy cell leukemia, immune suppression by deoxycoformycin and its consequences should clearly be a major consideration.

PROPOSED COURSE

This trial has been closed to further patient entry but remains open for follow-up of patients previously treated.

PUBLICATIONS

Nerenstone S, Martin A, Urba W, Lawrence J, Clark J, Smith J, Crum E, Miller R, Schoenberger C, Hawkins M, Longo D, Steis R. Treatment of hairy cell leukemia (HCL) with alternating cycles of 2' deoxycoformycin (DCF) and recombinant alpha-2a interferon (IFN). In: Leventhal B, ed. Proceedings of the American Society

of Clinical Oncology. New Orleans: American Society of Clinical Oncology, 1988;7:(Abst. 679)176.

Urba WJ, Baseler MW, Kopp WC, Steis RG, Clark JW, Smith JW, Coggin DL, Longo DL: Deoxycoformycin-induced immunosuppression in patients with hairy cell leukemia. Blood 1989;73:38-46.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09298-04 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Adoptive Chemotherapy with LAK Cells and IL-2

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Sznol	Senior Investigator	CTEP, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	L. A. VanderMolen	Medical Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba, L. C. Hartmann); Frederick Memorial Hospital, Frederick, MD (J. Hursey)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interleukin-2 in combination with ex vivo activated and adoptively transferred cells results in response rates of 20% in patients with renal cell cancer and melanoma. Preclinical studies show that cyclophosphamide and adriamycin can synergize with IL-2 to increase tumor responses. Interferon alfa alone has in vitro anti-proliferative properties and in clinical trials has produced response rates of 15-20% in patients with melanoma and renal cell cancer. We administered cyclophosphamide and adriamycin 2 days before IL-2 and infusion of lymphokine activated killer cells in a standard continuous infusion IL-2/LAK regimen. This was followed sequentially by single agent interferon-alfa. Two dose levels of IL-2 were used (3 and 6 mu/m2/d). Overall response rates were 22% in melanoma (9/41) and 14% in renal cell cancer (3/22). The higher IL-2 dose resulted in substantial, dose-limiting pulmonary toxicity when LAK cells were administered. The higher IL-2 dose appeared to be more active in renal cell cancer patients. However we conclude that the addition of chemotherapy and interferon-alfa to IL-2/LAK did not substantially improve response rates in patients with melanoma and renal cell cancer.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Senior Investigator	CTEP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Louis A. VanderMolen	Medical Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, NCI

OBJECTIVES

To increase the antitumor activity of a regimen employing continuous infusion IL-2 with adoptively transferred LAK cells by a) administering immunomodulatory doses of chemotherapy and b) sequencing treatment with a second biologic agent possessing antitumor activity, interferon-alfa.

METHODS EMPLOYED

Patients considered eligible for this study are evaluated in the outpatient clinic. If all eligibility criteria are met they are admitted to the study. Patients receive IL-2 by continuous infusion for 5 days (priming) (days 0-5). Dose levels of 3 and 6 million units/m^2/d were used in this study. Patients underwent leukapheresis on days 7-9 (reduced to days 7 and 8 only after approximately 15 patients were treated at the higher IL-2 dose level), and the cells were cultured in vitro with IL-2. Cyclophosphamide 300 mg/m^2 and adriamycin 25 mg/m^2 were administered on day 9. Beginning on day 11 IL-2 was again given by continuous infusion for 5 days. Cells obtained on day 7, 8 and 9 were infused on days 11, 12 and 14, respectively. Beginning day 21 patients received interferon-alfa 12 mu/m^2 TIW for a total of 9 doses, and were then evaluated for antitumor response. Patients with evidence of tumor regression were retreated. Beginning 7 days prior to receiving priming, and continuing through the last dose of IL-2, patients were treated with indomethacin and cimetidine.

MAJOR FINDINGS

Twenty-two melanoma patients were entered at the 3 mu/m^2/d dose level. Four patients were considered responders. Lymph node, lung, liver, skin and an axillary mass containing metastatic disease responded. Three of the responders progressed after receiving 3, 3, and 2 complete cycles. Twenty-two melanoma patients were entered at the 6 mu/m^2/d dose level. Two patients are too early to evaluate for response, and 3 died of treatment-related toxicity. Of the remaining 17 patients, 5 responded (complete response in subcutaneous sites, partial responses in a large pelvic mass, lymph node, and lymph node and lung metastatic sites, respectively). One other patient had evidence of tumor regression not meeting partial remission status and is continuing on treatment.

Nine patients with renal cell cancer were entered at the 3 mu/m^2/d dose level. One of these patients had a minor response, however, when retreated at the 6 mu/m^2/d dose level converted to a partial response. This patient had almost complete disappearance of all lung nodules. An additional 14 patients with renal cell cancer have been entered at the 6 mu/m^2/d dose level. One patient never received IL-2. Two patients are partial responders, one having achieved almost complete disappearance of all lung nodules. One patient is off treatment with stable disease, and three others have evidence of tumor regression and are continuing on treatment.

Overall 9/41 (22%) patients with metastatic melanoma and 3/22 (14%) with renal cell carcinoma (evaluable patients) responded. However, some patients remain on treatment and the actual response rates when the study matures may be higher. These figures do not represent substantial improvements over response rates reported for IL-2/LAK in the literature. Although no dose response relationship was demonstrated in melanoma, a firm conclusion regarding the utility of the higher dose must await maturation of response duration data. The higher dose of IL-2 does appear to be more active in patients with renal cell cancer although the statistical validity of this conclusion has not been proven.

There is clearly a dose-response relationship with regard to toxicity. Increased pulmonary and renal toxicity was apparent at the higher dose. Pulmonary toxicity was particularly evident with transfusion of LAK cells and required de-creasing the total number of LAK infusions from three to two. Three patients died (of 44 receiving 6 mu/m^2/d at any time).

PROPOSED COURSE

We plan to accrue a total of 25 patients with renal cell cancer at the 6 mu/m^2/d dose level. This will establish a phase II response rate with reasonable accuracy. Completion of accrual is expected before October 1989. A modified treatment approach is planned for melanoma patients to increase overall response rates. Future protocols will explore other effector cells (Anti-CD3 activated killer cells) and LAK/IL-2 in combination with melanoma-specific monoclonal antibodies.

PUBLICATIONS

Sznol M, Clark J, Smith J, Steis R, Urba W, VanderMolen L, Hartmann L, Hursey J, Markus S, Creekmore, S, Longo D. A pilot evaluation of interleukin-2 (IL-2) and lymphokine activated killer (LAK) cells in combination with chemotherapy and alpha-interferon. In: Leventhal B, ed. Proceedings of American Society of Clinical Oncology. San Francisco: American Society of Clinical Oncology 1989;8:(Abst. 742)91.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09305-03 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
2' Deoxycoformycin in Patients With HCL or T-Gamma Lymphoproliferative Disorder

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. G. Steis	Medical Officer	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (N. Englar); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study of deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disorder was written as a salvage protocol to treat those patients failing low-dose alfa interferon on other ongoing CRB protocols. Deoxycoformycin is known to have significant activity in hairy cell leukemia and has been reported to produce high complete response rates in previously untreated patients and to lead to responses in patients failing prior interferon therapy. It has unproven efficacy in the T-gamma lymphoproliferative disorder. Seven patients have been admitted to this study, four with hairy cell leukemia and three with T-gamma lymphoproliferative disorder. All four patients with hairy cell leukemia were refractory to interferon alfa-2a therapy. Three of these four patients had a substantial partial response to deoxycoformycin therapy and have at this moment only a minimal residual hairy cell infiltrate in the bone marrow. They have normalized their peripheral blood counts, and all have been off of deoxycoformycin therapy for a minimum of eighteen months; all remain in stable partial remission off all therapy. One patient with hairy cell leukemia died after the second dose of deoxycoformycin because of profound thrombocytopenia and refractoriness to HLA-matched platelet transfusions and extensive gastro-intestinal bleeding. At autopsy there had been a substantial reduction in the number of hairy cells in this patient's bone marrow. Of three patients with T-gamma lymphoproliferative disorder, one patient was a partial responder, and two patients were nonresponders. The one partial responder was an elderly woman with severe granulocytopenia. We conclude that deoxycoformycin has activity in interferon-resistant hairy cell leukemia and in some patients with T-gamma lymphoproliferative disorder. More patients will be treated.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. To determine the clinical effects of low doses of 2'deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease refractory to or intolerant of low-dose alfa interferon.

2. Determine the biochemical consequences of the administration of 2'deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease.

3. To determine the immunologic consequences of the administration of deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease.

4. To study the effects of this therapy on the serum level of a soluble form of the IL-2 receptor.

METHODS EMPLOYED

Patients with hairy cell leukemia on our ongoing trial of single-agent alfa interferon are eligible for this study if they have progressive disease during the course of interferon administration. Patients with hairy cell leukemia are also eligible for the study if the side effects of alfa interferon are intolerable. Patients with T-gamma lymphoproliferative disorder are eligible for this study if they have not responded to three consecutive months of administration of alfa interferon.

Once patients are deemed eligible for the study, they receive 4 mg/m^2 of deoxycoformycin intravenously every week for three consecutive weeks. Patients then receive 4 mg/m^2 on alternating weeks. Patients will receive this therapy for a minimum of 12 weeks, and if disease has stabilized, continued therapy will be given for at least 6 months unless disease progression or prohibitive toxicity occurs. If a patient achieves a pathologically documented complete remission, therapy will be continued for three more months beyond the time of complete remission. If patients have a partial response to therapy, therapy may be continued indefinitely unless serial biopsies show persisting stable amounts of disease in the bone marrow. Patients who have less than a partial response will be taken off of therapy at the six month time point. Deoxycoformycin is administered after hydration with 1 liter of D5 and half normal saline. After administration of the deoxycoformycin, one further liter of fluid is given intravenously.

MAJOR FINDINGS

Four patients with hairy cell leukemia refractory to interferon alfa-2a therapy were treated on this study. All four of these patients were found to have neutralizing anti-interferon alfa-2a antibodies that developed in association with the development of clinical resistance to interferon alfa-2a. All four patients had a very rapid reduction in the peripheral blood hairy cell count and some myelosuppression induced by the deoxycoformycin. One patient died after two doses of deoxycoformycin due to persistent severe gastrointestinal bleeding associated with profound thrombocytopenia which was worsened by deoxycoformycin therapy but was present prior to institution of treatment and was related to the underlying hairy cell leukemia. The other three patients completed six months of therapy with deoxycoformycin and at the time of post-therapy clinical evaluation were found to have minimal numbers of hairy cells in the bone marrow. Peripheral blood counts in all cases had normalized. These patients have been off of treatment for a minimum of eighteen months and all remain in a stable partial remission. There has been no progressive increase in the number of hairy cells in the bone marrow in any of these patients since the end of treatment.

Among three patients with the T-gamma lymphoproliferative disorder, there has been one partial response and two patients with no response. The patient with the partial response was an elderly female with severe granulocytopenia and a history of recurrent infections. After institution of deoxycoformycin, there was a progressive increase in the peripheral blood granulocyte count and a progressive clearing of T-gamma cells from the bone marrow and peripheral blood. After approximately two months of therapy the patient developed a left cerebral vascular accident and was removed from therapy. Bone marrow biopsies at this time showed that she had partial clearing of T-gamma cells from the bone marrow.

We have evaluated immune function in hairy cell leukemia patients following deoxycoformycin therapy. Immune function during the course of interferon treatment prior to institution of deoxycoformycin therapy was essentially normal. With institution of deoxycoformycin therapy, however, there was a progressive and marked decrease in the number of T lymphocytes in the peripheral blood. This was associated with marked decreases in proliferative responses of the peripheral blood mononuclear cells, inversion of the T4/T8 ratio, and a decrease in the number of CD4 positive lymphocytes to less than 200 cells per cubic millimeter. None of these patients developed opportunistic infections, but they have not recovered immune function as of this date. We continue to evaluate these patients for the duration and clinical consequences of this immune-suppression.

Toxicities of deoxycoformycin in the setting of T-gamma lymphoproliferative disorder or advanced refractory hairy cell leukemia included myelosuppression, possibly contributing to a febrile episode in one patient, gastrointestinal hemorrhage secondary to severe thrombocytopenia in one patient, anorexia, nausea, and vomiting, mild skin rash, fatigue, and chills. One patient had a pulmonary embolus felt to be secondary to a central venous catheter, and one patient had a cerebral vascular accident that occurred during placement of a central venous line. Whether this was a consequence of line placement or was incidental is impossible to determine.

SIGNIFICANCE

For patients with hairy cell leukemia, recombinant interferon alfa has been a highly successful therapy. It is clear, however, that between 5 and 10 percent of patients with hairy cell leukemia do not respond to interferon alfa and perhaps an additional 10 percent of patients will acquire resistance to interferon alfa usually in association with anti-interferon antibodies. Optimal treatment for this subset of patients has not been defined but it appears from small numbers of patients reported in the literature that deoxycoformycin therapy is highly effective in this setting. Our results so far confirm previously published results from the BRMP and elsewhere but also indicate that great care should be used in administering this therapy because of its propensity to worsen pre-existing cytopenias. The immune suppression induced by deoxycoformycin in patients with hairy cell leukemia was not unexpected though the severity and duration of immune suppression was surprising.

The current treatments for patients with T-gamma lymphoproliferative disorder are unsatisfactory. Corticosteroids, cytotoxic drugs, and splenectomy have been tried with mixed and minimal success. Deoxycoformycin was used in an attempt to treat these patients because of its previously documented effects on normal T-cell function and because of its known activity in childhood acute lymphoblastic leukemia. Although we have only treated a small number of patients, it is clear that this drug has some activity in this disease. We are aware of no other studies using deoxycoformycin in this disease, and we plan to continue to evaluate the effectiveness of this therapy in this rare leukemia.

PROPOSED COURSE

This protocol is being kept open for further patient accrual. In addition to evaluating this drug for efficacy, we intend to carefully monitor immune function in those patients receiving deoxycoformycin and determine the long-term consequences of the immunosuppression observed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09306-03 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phase Ib Trial of Intraperitoneal GM-CSF

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. G. Steis	Medical Officer	CRB, NCI
Others:	J. Janik	Expert	CRB, NCI
	J. W. Smith	Senior Staff Fellow	CRB, NCI
	D. L. Longo	Associate Director	OAD, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (R. Barney); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monocytes and macrophages can be activated with a variety of cytokines to kill tumor targets in vitro. A variety of animal studies have also suggested that activation of monocytes and macrophages in vivo can bring about tumor responses in selected model systems. A previous study at the BRMP suggested that human monocytes harvested from the peripheral blood can be activated in vitro and adoptively transferred into the peritoneal cavity of patients with peritoneal carcinomatosis and that this approach to the treatment of cancer may have limited efficacy. In this study we will administer recombinant human granulocyte macrophage colony stimulating factor (rHuGM-CSF) intraperitoneally to patients with disease limited to the peritoneal cavity. Previous studies in mice have suggested that administering GM-CSF in this fashion will result in the recruitment of large numbers of monocytes and macrophages into the peritoneal cavity. If we can accomplish this in humans we can test the hypothesis that monocytes and macrophages can bring about tumor responses in humans. In three separate parts of this study patients will receive either GM-CSF alone, GM-CSF with interferon gamma, or GM-CSF with interleukin-2 (IL-2). All drugs will be administered intraperitoneally. Two patients have been enrolled and treated so far. We have seen substantial increases in the number of monocytes and granulocytes in the peritoneal fluid. No tumor responses have yet been observed. Thus, it has been determined that monocytes can be recruited to the peritoneal cavity. Additional patients and treatments will be required before activation of monocytes in vivo and antitumor activities in vivo can be assessed.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
John Janik	Expert	CRB, NCI
John W. Smith	Senior Staff Fellow	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. Determine the toxic effects of intraperitoneal injections of rHuGM-CSF alone or in combination with interferon gamma or with IL-2.

2. To determine the effects of intraperitoneal injections of rHuGM-CSF alone or with interferon gamma or IL-2 on peripheral blood and peritoneal monocyte and granulocyte number and function.

3. To determine, in a preliminary fashion, the antitumor effects and pharmacokinetics of intraperitoneally administered rGM-CSF alone or with interferon gamma or IL-2.

METHODS EMPLOYED

Patients with tumor limited to the peritoneal cavity are eligible for this study. Although most patients are expected to have either ovarian or colon carcinoma, any patient with disease limited to the peritoneal cavity is eligible. Patients must also have an adequate performance status, a potentially patent peritoneal space, have received or refused to receive standard therapy of proven survival benefit, and adequate physiologic function.

This study is divided into three parts. In Part 1 rHuGM-CSF is administered intraperitoneally every 8 hours for 5 consecutive days at increasing doses. After one week rest, patients will again receive 5 consecutive days of intraperitoneal GM-CSF. Samples of blood and peritoneal fluid will be obtained serially and cell counts and differentials determined. Assays of monocyte and granulocyte activation such as enhanced expression of HLADR or Fc receptors, and hydrogen peroxide generation will be performed serially. If no tumor response is observed after GM-CSF alone, patients are eligible to proceed to Part 2 of the study where GM-CSF will be given intraperitoneally with interferon gamma 5 days per week for 3 consecutive weeks. The dose of GM-CSF will be escalated in groups of 3 patients but interferon gamma will be given at a constant dose of .05 mg/m2. Again, serial samples of blood and peritoneal fluid will be obtained to monitor cell numbers and activation. If patients show no response to GM-CSF and interferon gamma, they may progress to Part 3 where GM-CSF will be given with IL-2 intraperitoneally. Treatments will be given at escalating doses of GM-CSF but with a constant dose of IL-2. Again, cell number and activation assays will be performed.

Tumor responses in this study will be assessed by either serial CT scans or peritoneoscopy.

MAJOR FINDINGS

To date only 2 patients have been entered on study. One patient completed all ten days of GM-CSF infusion intraperitoneally in Part 1 of the study. This patient was found to have a marked increase in the cell counts in the peritoneal cavity with significant increases in the number of macrophages and monocytes. The results of assays of activation of these cells are pending. The patient had no toxic effects during the course of administration of GM-CSF except for low-grade fever and palmar erythema. The second patient developed an acute subendocardial myocardial infarction on the third day of intraperitoneal injections of GM-CSF. Although the patient had low-grade fever on days 1 and 2 of the GM-CSF infusions, he was afebrile and under no apparent stress at the time chest pain developed. Subsequent angiogram revealed the presence of coronary artery disease in a distribution consistent with his myocardial infarction. In this patient, large numbers of monocytes and granulocytes were observed to infiltrate the peritoneal cavity following i.p. infusions of GM-CSF.

Thus, we have answered one major question posed by our study. GM-CSF given intraperitoneally is capable of bringing about a substantial increase in the number of cells in the peritoneal cavity. These cells are known to have the potential to bring about antitumor responses and we intend to continue to give GM-CSF alone and in combination with IL-2 or interferon gamma.

SIGNIFICANCE

A major potential limitation of adoptive immunotherapy of cancer is suboptimal trafficking of ex vivo activated cells following their i.v. administration. Monocytes activated ex vivo with interferon gamma and administered intravenously were observed to traffick to endothelial surfaces in an earlier study conducted at the BRMP. If activated monocytes are to bring about tumor responses, an alternative method for delivering these activated cells to sites of disease must be developed. In this study, we have taken advantage of the ability of GM-CSF to act as a chemotactic agent for monocytes and have administered it intraperitoneally to patients with cancer limited to this anatomic compartment. The preliminary results so far suggest that it is possible to recruit cells to the site of the tumor. What remains is to be determined is if these cells are activated with GM-CSF alone and if they can be activated to a greater extent with the coadministration of either interferon gamma or IL-2.

PROPOSED COURSE

This study remains open for further patient entry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09308-03 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phase II Efficacy Study of Roferon A (RO22-8181/002) in Hairy Cell Leukemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. G. Steis	Medical Officer	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study is an ongoing study of the efficacy and toxicity of interferon alfa-2a in hairy cell leukemia. Although active in this disease, interferon rarely brings about complete remissions and relapse and/or disease progression is the rule after discontinuation of treatment. In addition to evaluating initial response to therapy, we continue to administer interferon to responding patients to determine the long-term efficacy of this treatment. The initial therapeutic activity of interferon has been confirmed. Of 53 patients evaluable for response (of a total of 56 entered), there was 1 complete responder, 34 partial responders, 6 minor responders, 7 patients with stable disease and 1 patient with progressive disease. Minor responses in this disease are clinically meaningful responses in that peripheral blood counts returned to essentially normal levels. Thirty-four patients continue on therapy for a median therapy duration of 45 months. Thirty continue to receive interferon at the standard dose of 3 million units three times weekly but 4 patients have required dose escalations to 3 or 6 million units daily to maintain response. A total of 20 patients have been removed from interferon treatment for a variety of reasons, the most common of which has been disease progression on therapy. Disease progression during interferon therapy has been correlated with the development of neutralizing interferon antibodies. Longer follow-up will be necessary to determine if this form of continuous therapy is associated with better long-term disease control than intermittent therapy.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

To evaluate the safety and efficacy of recombinant interferon alfa in patients with hairy cell leukemia.

METHODS EMPLOYED

Patients to be considered eligible for this study must have histologically documented hairy cell leukemia and one or more cytopenias. At the initiation of therapy, all patients must have a platelet count of less than 100,000 per cubic millimeter and/or a granulocyte count of less than 1,500 per cubic millimeter and/or a hemoglobin of less than 10 grams percent or transfusion dependency. After their initial clinical evaluation, patients are then given 3 million units of recombinant alfa interferon subcutaneously daily for 4 to 6 months. In responding patients, maintenance therapy is given at a dose of 3 million units subcutaneously 3 times per week. Responding patients have continued on therapy indefinitely. Patients who experience disease progression are given escalated doses of recombinant alfa interferon in an attempt to control their disease. If dose-limiting side effects develop at the higher doses, patients are taken off study.

MAJOR FINDINGS

Fifty-six patients have been entered on this study of whom 53 are evaluable for response. One inevaluable patient was found to have glioblastoma multiforme, one died of a Rhizopus brain abscess, and one was not evaluable for hematologic response because she had painful infiltration of hairy cells into bone but normal peripheral blood counts. The initial responses included one complete remission, 34 partial remissions, six minor remissions, eleven patients with stable disease and only one patient with disease progression during therapy. There was no difference in response rate between previously splenectomized patients (30 patients) and patients who had not previously undergone splenectomy (23 patients). The therapy was well tolerated with only minimal interferon related side effects including fatigue, chills, myalgias, fever, anorexia, weight loss, asymptomatic elevations in liver function tests, minor alopecia, and minor impotence.

A total of 20 patients have been removed from treatment. Reasons for discontinuation of therapy included failure to respond initially (1), disease progression after initial response (6), myocardial infarction (1), squamous cell carcinoma of the lung (2), erythrocytosis (1), noncompliance (1), patient refusal (2), depression (1), impaired cognition (2), fatigue (1), sensory

neuropathy (1), and a worsening of a rheumatic syndrome (1). The major reason for removal from study was because of the development of interferon resistance. We evaluated serial serum specimens in our patients and found a correlation between the development of neutralizing interferon antibodies and disease progression. Of 16 patients who developed neutralizing antibodies, 6 developed interferon resistance; 15 patients developed only non-neutralizing antibodies and none of these patients had clear cut interferon resistance. Importantly, of 20 patients who did not develop interferon antibodies, none showed interferon resistance. The association of interferon resistance with the presence of neutralizing interferon antibodies is significant ($p2 = 0.008$). Interestingly, the neutralizing interferon antibodies found in this study neutralized only recombinant interferon alfa-2a; when these sera were tested for their neutralizing ability against a preparation of partially purified natural interferon, no neutralizing activity was found. Therefore, if these antibodies do mediate interferon resistance, this resistance could in theory be overcome by the administration of partially purified natural interferon. The development of neutralizing interferon antibodies was observed in all patients who developed acquired interferon resistance; we did not observe acquired interferon resistance in any patient in the absence of neutralizing interferon antibody.

As of this date, 34 patients continue to receive interferon alfa; 30 patients continue therapy at the standard dose of 3 million units 3 x weekly; four patients required dose escalations to 3 or 6 million units daily to maintain their initial response. All patients who required dose escalation to maintain response also had neutralizing interferon antibodies.

SIGNIFICANCE

In this study we have therefore confirmed effectiveness of interferon in the initial management of patients with this disease. The issue at hand currently is the optimal management of patients in the long-term. Complete remissions after interferon therapy are rare and disease progression after discontinuation of interferon is the rule. After obtainment of an initial response, some form of subsequent therapy will be needed. One could administer continuous therapy as we are, or administer substantially (10 fold) lower doses or administer therapy intermittently based upon the peripheral blood counts and/or clinical status of the patient. Unfortunately, we do not have enough patients to randomize them between these three treatment alternatives. The results of our study in which "induction" doses of interferon are given continuously will be compared with other studies giving interferon intermittently or at lower doses after obtainment of the initial response to determine the optimal long term management of these patients.

PROPOSED COURSE

This study has been closed to new patient entry but is being kept open to provide drug for patients continuing to receive therapy on this study. Patients will be followed closely for the development of late toxic effects, the development of interferon antibodies, the development of interferon resistance, and the development of other late complications of therapy to help determine whether chronic maintenance therapy with interferon is the most appropriate form

of therapy to administer to these patients following an initial course of induction therapy.

PUBLICATIONS

Steis RG, Marcon L, Clark J, Urba W, Longo DL, Nelson DL, Maluish AE. Serum soluble IL-2 receptor as a tumor marker in patients with hairy cell leukemia. Blood 1988;71:1304-9.

Steis RG, Smith II JW, Urba WJ, Clark JW, Itri LM, Evans LM, Schoenberger C, Longo DL. Resistance to recombinant interferon alfa-2a in hairy-cell leukemia associated with neutralizing anti-interferon antibodies. N Eng J Med 1988;318:1409-13.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09314-02 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Autologous Activated Monocytes Given I.P. for Colonic Carcinomatosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, NCI
	P. Miller	Biologist	OAD, NCI
	L. VanderMolen	Medical Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*
Nuclear Medicine, Clinical Center, NIH, Bethesda, MD (J. Carrasquillo); Program Resources, Inc., Frederick, MD (W. J. Urba, L. Hartmann); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)
- [X] (a) Human subjects
- [X] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Isolated and purified human monocytes have been shown to be capable of killing tumor targets in vitro following activation by interferon-gamma. The purpose of this study is to determine if autologous activated monocytes can be used as an effective form of adoptive immunotherapy for colon cancer patients with disease limited to the peritoneal cavity. This protocol is based on a previous BRMP protocol which employed a similar study design; however, the patient population had undergone laparotomy and complete surgical excision of all macroscopic tumor. The patients in that protocol tolerated leukapheresis and infusions of activated monocytes along with interferon-gamma with tolerable toxicity. This study will enroll patients who have measurable macroscopic disease to determine whether adoptively transferred activated monocytes have cytotoxic activity against established tumor. Because the disease is limited to the peritoneal cavity, the infused monocytes will have adequate contact with the tumor deposits, and questions of trafficking should not complicate the analysis of their efficacy.

One patient was entered onto this study during the last year. Protocol therapy was well tolerated with only Grade I fever, chills, abdominal pain, myalgia and fatigue. Although we were able to demonstrate anti-tumor cytotoxicity of the peritoneal monocytes in vitro, the patient had evidence of progressive disease when she was re-evaluated after 8 weeks of therapy. The protocol was closed this spring because of poor patient accrual and because the company supplying the interferon gamma decided to support only one clinical investigation of intraperitoneally administered interferon gamma.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
Paul Miller	Biologist	OAD, NCI
Louis VanderMolen	Medical Staff Fellow	CRB, NCI

OBJECTIVES

1. To measure the clinical effects including antitumor response rate of intraperitoneal infusion of autologous cytotoxic monocytes activated and boosted with recombinant interferon-gamma in patients with peritoneal colorectal carcinomatosis.

2. To determine the tumoricidal activity in vitro of monocytes removed from the peritoneal cavity of patients following intraperitoneal infusion and daily in vivo boosting with interferon-gamma.

3. To monitor monocyte trafficking of cells following intraperitoneal infusion using ^{111}indium-labeled monocytes and whole body scanning.

METHODS EMPLOYED

The criteria for patient selection are:

1. Patients must have biopsy proven colorectal carcinoma with peritoneal metastases.

2. Patients must have macroscopic disease that is measurable or evaluable either on CAT scan of the abdomen or at peritoneoscopy or laparotomy. Patients with metastases outside the peritoneal cavity are not eligible.

3. Patients must have a Karnofsky performance status of greater than 70 percent, a life expectancy greater than six months, and must not have received any anti-neoplastic therapy for at least one month prior to the study.

4. Patients must have adequate hematologic, hepatic, renal, cardiac, and pulmonary function.

5. Patients may not have received therapy with interferon-gamma previously.

Initial evaluation will consist of a history and physical, chest X ray, CAT scans of the chest, abdomen, and pelvis, urinalysis, CBC, Chem 20, EKG, CEA, hepatitis B surface antigen, and HIV antibody. In addition, all patients will undergo peritoneoscopy or laparotomy prior to the start of treatment at

which time a Tenckhoff catheter will be inserted and multiple biopsies will be obtained.

One week after the insertion of a Tenckhoff catheter patients will undergo cytapheresis after which monocytes will be purified by countercurrent elutriation and cultured with recombinant interferon gamma overnight. The following day, these activated monocytes will be infused into the patient along with interferon gamma 1,000 units/ml in two liters of dialysis fluid. Patients will receive daily infusions intraperitoneally of interferon gamma in dialysis fluid. Cytapheresis and monocyte infusions will be repeated weekly for a total of eight infusions unless disease progresses during this time. During the middle of therapy, patients will be infused with radiolabeled monocytes and imaged at the Nuclear Medicine Department, NIH. At the end of eight weeks of therapy, the patients will have a repeat peritoneoscopy to assess response to treatment. Patients without progressive disease will receive eight more weeks of identical therapy followed by repeat evaluation. At sixteen weeks, therapy will stop for all patients who have had progressive disease or no more than stable disease. Patients with a partial response will receive identical therapy with repeat evaluations every eight weeks and continue therapy as long as they remain in partial response or for eight weeks after they achieve a complete response.

Each time the patient undergoes leukapheresis and monocytes are purified and activated, the in vitro antitumor activity of the isolated monocytes and interferon-activated monocytes will be assayed against human colon cancer cell lines. In addition, cells will be harvested from the peritoneal fluid via the Tenckhoff catheter periodically, and their cytotoxic function will also be assayed in vitro.

MAJOR FINDINGS

One patient was enrolled on this study during the last year. This patient tolerated protocol therapy as described above with only mild toxicities consisting of grade 1 fever, chills, pain, myalgia and fatigue. Repeat peritoneoscopy after 8 weeks of treatment showed clear-cut evidence of progressive disease and the patient was taken off the protocol. Between 200 to 500 million monocytes were infused intraperitoneally each week. Cytotoxicity against cancer cell lines was assayed in vitro. The patients' autologous monocytes that were activated ex vivo with gamma interferon and the ascites cells that were sampled after the treatment demonstrated anti-tumor cytotoxicity. The Indium labeled monocytes remained in the peritoneal cavity on whole body scan.

PROPOSED COURSE

This protocol has been closed because of poor patient accrual and because of the refusal of the company supplying the interferon gamma to support more than one clinical trial using intraperitoneally administered interferon gamma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09324-02 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antitumor and Immunological Correlates of Individualized IL-2 Administration

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. P. Creekmore	Chief	BRB, NCI
Others:	J. Janik	Expert	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Frederick Memorial Hospital (N. Engler); Program Resources, Inc., Frederick, Maryland 21701 (W. J. Urba, J. Beveridge); Cancer Therapy Evaluation Program (M. Sznol).

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This trial was designed to investigate the antitumor and immunological effects of chronic IL-2 administration. In previous BRMP-sponsored studies, induction and maintenance of endogenous LAK activity and antitumor responses have been produced by administering IL-2 alone with the treatment schedule and dosages used in this trial. This approach avoids the severe toxicity and complicated ex vivo laboratory manipulations of LAK/IL-2 regimens. In this investigation, IL-2 is given by 24-hour continuous infusion twice weekly for up to 4 weeks, at a dose of 3×10^7 U/m2 (BRMP units) per 24-hour infusion. Subsequent IL-2 doses are adjusted in the individual patient to sustain high circulating levels of Leu19 positive cells. At the end of 3 months of treatment tumor response is assessed. Modest evidence of antitumor effect has been seen (1 PR, 1 minor response in 14 patients). Patients achieved and maintained high Leu19 levels and increases in circulating NK and LAK activity. Patients also demonstrated bimodal Leu-19+ populations, consisting of CD16+/Leu19 dim and CD16-/Leu19 bright subpopulations. LAK activity and Leu19 levels have been sustained in most patients in the outpatient phase of treatment suggesting that combination treatments may be designed around this IL-2 regimen, involving other BRMs, chemotherapy, monoclonal antibodies, etc., which might exploit or enhance the sustained levels of circulating cytotoxic cells for greater antitumor response. A follow-on trial now employs moderate dose of cyclophosphamide administered once monthly to reduce suppressor cell populations. In the second trial, patients undergo leukapheresis at monthly intervals in order to obtain cell numbers sufficient to perform cell sorting studies to characterize the circulating Leu19+ populations.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Stephen P. Creekmore	Chief	BRB, NCI
John Janik	Expert	CRB, NCI
J. W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
R. G. Fenton	Expert	CRB, NCI

OBJECTIVES

The objectives of this study are:

1. To determine if a high sustained level of circulating LAK cells can be generated in patients using an individualized twice-weekly IL-2 regimen which can be administered to outpatients.

2. To determine the long-term immune modulatory effects of IL-2 in such a regimen.

3. To determine the antitumor effects of such a regimen.

4. To determine the toxicity of such a regimen.

5. To investigate mechanisms of antitumor effects by serial tumor sampling in selected patients.

METHODS EMPLOYED

Patients are required to have measurable tumor, good performance status (70% Karnofsky or greater), expected survival greater than 3 months, and no brain metastases. Tumor types are renal cell carcinoma, malignant melanoma, or other tumor cell types at the discretion of the principal investigator.

Patients are treated with recombinant IL-2 (Hoffmann-LaRoche) in the following regimen:

3-4 weeks high-dose induction: IL-2 at 3 x 10^7 U/m^2 by 24 hour iv infusion b.i.w. (inpatient). Target Leu19 level: 2500/cu mm.

Maintenance phase treatment: IL-2 at 3 x 106 U/m2 by 24 hour iv infusion b.i.w. (outpatient).

The dosage of IL-2 to be administered during the maintenance phase is adjusted to maintain Leu19 levels above 1000/cu mm or 20% of the maximum level attained after induction (whichever is higher). Doses greater than or equal to 10^7 U/m^2 are given only to inpatients, and during maintenance therapy patients may receive up to four high-dose treatments per month.

Patients are assessed for response after 3 months.

Stable or responding patients continue on therapy up to 1 year.

MAJOR FINDINGS

Seventeen patients were entered on the initial trial. Fourteen completed the induction period and achieved the target Leu19 cell level. Three were removed from study before completing induction (1 for PVCs, 1 for marked tumor progression, 1 refused treatment). One partial (renal cell) and one minor response (ovarian cancer) were seen. Significant toxicity during induction included infections related to intravenous catheters, hypotension requiring i.v. fluid administration and occasional use of pressors at the higher inpatient dose levels, and other usual IL-2-related toxicities.

All patients completing induction achieved boosts in Leu19 levels and circulating LAK and NK activity. These immune effects were sustained within the target range in most patients in the outpatient phase of treatment.

In the follow-on trial employing moderate doses (300 mg/m2) of cyclophosphamide, 8 patients have been entered on study. Toxicity has been similar to the original regimen. Neutropenia possibly related to cyclophosphamide was seen in 2 heavily pretreated patients. As with the original version, boosts of NK, LAK and Leu19 levels have been seen. Patient accrual is continuing. One patient with melanoma has shown a minor response.

SIGNIFICANCE

This trial has demonstrated that the IL-2 regimen described can generate and sustain large numbers of circulating Leu19+ cells. Modest antitumor activity of the regimen alone is suggested by the clinical responses.

In general, these results indicate that combination treatments may be designed around this IL-2 regimen involving other BRMs, chemotherapy, monoclonal antibodies, etc., which might exploit or enhance the sustained levels of circulating cytotoxic cells for greater antitumor response.

The toxic effects of the maintenance regimen will allow addition of other treatment modalities. Follow-on trials will begin later this year using this regimen with the addition of monoclonal antibodies (recognizing determinants on colon cancer cells and melanoma) which mediate ADCC.

PROPOSED COURSE

The initial trial has been closed to further entry and has been reopened with a treatment modification to include moderate doses of cyclophosphamide to reduce suppressor cell populations. Related trials are also beginning using the same IL-2 regimen in combination with other agents (flavone acetic acid and poly-ICLC), and a follow-on trial has been written for the same IL-2 regimen plus an ADCC-mediating monoclonal antibody.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09325-02 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
A Phase I Study of Flavone-8 Acetic Acid in Combination with Interleukin-2

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. P. Creekmore	Chief	BRB, NCI
Others:	R. H. Wiltrout	Senior Investigator	LEI, NCI
	I. Green	Senior Investigator	BRB, NCI
	J. Janik	Expert	CRB, NCI
	J. W. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI

CONTINUED ON NEXT PAGE

COOPERATING UNITS *(if any)*
Frederick Memorial Hospital (K. Madera); Program Resources, Inc., Frederick, Maryland 21701 (W. J. Urba, J. Beveridge, R. Hornung); Cancer Therapy Evaluation Program, NCI (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This trial is designed to evaluate the immunomodulatory, toxic and antitumor effects of the combination of flavone-8 acetic acid (FAA) and interleukin-2 (IL-2). FAA as a single agent is curative for early and advanced murine colon 38 and has activity in several other murine solid tumor models. Interestingly, FAA has only modest direct cytotoxicity. In a murine renal cell carcinoma model, the combination of FAA + IL-2 is curative when neither FAA nor IL-2 alone at the same doses are active. Immunologic studies in mice and humans have shown that FAA boosts natural killer cell activity and increases serum interferon levels. FAA has been studied as a single agent in phase I clinical trials, without evidence of clinical activity.

Twenty-two patients have completed the trial. No consistent immunomodulatory or antitumor effects have been observed. FAA is a weak acid and, to avoid drug precipitation in renal tubules, was given after urinary alkalinization, a maneuver that was not employed in the animal studies. Subsequent animal studies showed ablation of the immunomodulatory and antitumor effects of FAA plus IL-2 when the animal's urine was alkalinized. The protocol is currently being amended to infuse the FAA over a shorter period of time and to delete urinary alkalinization.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Stephen Creekmore	Chief	BRB, NCI
Robert H. Wiltrout	Senior Investigator	LEI, NCI
Ira Green	Senior Investigator	BRB, NCI
Jeffrey W. Clark	Senior Staff Fellow	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
John Janik	Expert	CRB, NCI

OBJECTIVES

The objectives of this study are:

1. To explore the immunological effects and toxicity of FAA alone and in combination with IL-2.

2. To determine optimal dosage of FAA in combination with IL-2 for immune modulation, as measured by circulating immune effector cells, and where possible determinations of lymph node and tumor infiltrating lymphocyte populations.

3. To record the antitumor effects of the regimen in a preliminary patient sample.

4. To evaluate antitumor effects and mechanisms by serial tumor sampling in selected patients.

METHODS EMPLOYED

Patients are required to have metastatic cancer without an alternative therapy of known benefit, a life expectancy of at least four months, measurable or evaluable disease, and adequate renal, hepatic, hematologic, cardiac, and pulmonary function.

Patients are treated in 8 levels of 3 patients each. Patients are treated only at a single FAA and IL-2 dose level. FAA is given once weekly by a 6-hour intravenous infusion at doses of 8.5 g/m^2 or 10 g/m^2. IL-2 doses are: 1×10^6, 3×10^6, 1×10^7, or 3×10^7 U/m^2 and are given by a 24-hour intravenous infusion.

Each patient first receives FAA as a single agent weekly for two weeks. IL-2 is then introduced as a twice weekly infusion with the first dose starting 24 hours after the weekly dose of FAA.

Patients are monitored for toxicity and immunological changes following treatment with FAA alone and FAA with IL-2. Immune monitoring includes NK and LAK cell assays as well as cell surface marker penotyping. Patients are evaluable for clinical response after completing two weeks of treatment with weekly FAA plus twice weekly IL-2.

MAJOR FINDINGS

Twenty-two patients have been entered on study. Seven have been taken off study before completion of the trial, (3 brain metastases, 1 bowel obstruction, 2 at patient request, and 1 intrathoracic bleed with no temporal relationship to FAA). No clinical responses have been seen. Immune studies suggest no marked potentiation of IL-2 effects by FAA. Currently, patients are being accrued to the 2 highest dose levels in the dose-escalation of IL-2 and FAA.

Toxicities attributed to the regimen include grades 1-3 hypotension, grade 1-2 myalgia and weakness, increased CPK and LDH, increased template bleeding time, fatigue, diarrhea, fever, nausea, and anorexia. Toxicities have usually resolved within 24 hours after the infusion.

SIGNIFICANCE

The FAA/IL-2 synergy in animal experiments has not so far been replicated in humans. Further exploration of FAA schedules is planned.

PROPOSED COURSE

Animal studies suggest that the FAA/IL-2 synergy observed in a renal cell carcinoma model is optimal at high peak FAA levels. A follow-on trial has been written and submitted to the IRB, to explore shorter infusion times (1 and 3 hours instead of 6 hours by CI) of FAA in order to achieve higher peak levels of drug, in the hope of achieving the desired FAA/IL-2 synergy. Additionally, urinary alkalinization in the animal model ablated the immunomodulatory and antitumor effect of FAA and IL-2 and will be deleted from the revised protocol. Accrual to this modification of the trial will be undertaken when the 2 highest dose levels have been explored on the 6 hour CI schedule.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09329-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
rGM-CSF and High Dose Carboplatin Therapy in Refractory Ovarian Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. A. VanderMolen	Medical Staff Fellow	CRB, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI (M. Sznol); Medicine Branch, NCI (E. Reed)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This trial was designed to test the ability of rGM-CSF to protect patients with refractory ovarian carcinoma from the bone marrow suppressive effects of high dose carboplatin. Previous studies with high-dose carboplatin have demonstrated response rates of up to 27% in this cohort of patients; these antitumor effects were associated with severe and prolonged myelosuppression. In this trial, carboplatin was administered every five weeks at a dose of 800 mg/m2. Beginning three days after carboplatin administration, rGM-CSF was given at varying doses for 18 days to enhance the rate of bone marrow recovery. This study is being conducted jointly with the Medicine Branch, NCI. So far at the BRMP five patients have been entered. All had significant myelosuppression following high dose carboplatin and all three patients currently beyond their first cycle of therapy required significant dose reductions. All patients required platelet transfusions and hospital admission for treatment of fever in association with granulocytopenia. Whether GM-CSF has resulted in a shortening of the duration of granulocytopenia cannot be determined from this study. However, thrombocytopenia has been severe and prolonged and appears not to have been affected by GM-CSF. One of the patients treated at the BRMP is evaluable for response and achieved a partial response. The current trial will be continued until sufficient numbers of patients have been accrued to determine whether toxicity is sufficiently reduced by the addition of rGM-CSF to warrant a Phase III trial comparing carboplatin and rGM-CSF to carboplatin alone.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Louis A. VanderMolen	Medical Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. To determine if rGM-CSF alters the pattern of myelosuppression associated with high dose carboplatin (800 milligrams/m2) in patients with refractory ovarian carcinoma.

2. To determine if the dose intensity and/or cumulative dosage of carboplatin may be increased in association with rGM-CSF administration.

3. To compare the response rate of high-dose carboplatin with rGM-CSF to historical controls treated without rGM-CSF.

4. To evaluate the toxic effects of high-dose carboplatin and rGM-CSF.

METHODS EMPLOYED

Patients entering this trial must have a histologic diagnosis of an epithelial ovarian carcinoma refractory to prior therapy. Prior therapy may include cisplatin unless doses of greater than or equal to 150 mg/m^2 were administered without response. Patients must also have adequate bone marrow, hepatic and renal function with serum creatine levels less than 1.5, white blood cell counts greater than 3,000 per cubic millimeter and platelet counts greater than 50,000 per cubic millimeter, and SGOT and SGPT levels less than 100 units/ml.

After an initial staging evaluation, patients are administered carboplatin at a starting dose of 800 mg/m^2 every 35 days. Recombinant GM-CSF is given as a subcutaneous injection starting 72 hours after carboplatin and is given for a minimum of 21 days or until the total white blood cell count is above 2,000 for seven consecutive days. GM-CSF dose levels include 3, 10, 20, and 30 micrograms per kilogram per day. Antitumor effects are evaluated after four cycles of high-dose carboplatin and GM-CSF therapy. After 15 patients are treated, the trial will be analyzed and compared to the results obtained in historical control treated under similar circumstances but with lower carboplatin doses without GM-CSF.

MAJOR FINDINGS

Five patients have been admitted to this study at the BRMP. One patient has completed three cycles, a second two cycles and the three remaining patients are in various stages of the first cycle of therapy. None of the patients have reached four cycles of therapy for re-evaluation. The most frequent

side effect seen in the patients treated with high-dose carboplatin in conjunction with rGM-CSF has been thrombocytopenia. The thrombocytopenia has been in general refractory to random donor platelet administration and in two of the patients who received HLA-matched platelets responses have been variable. The platelet nadirs have been prolonged and severe with counts below 10,000 and evidence of hemorrhage typically manifested by petechia, hematuria, epistaxis or gastrointestinal hemorrhage. Patients have frequently required administration of packed red blood cells although there have been no massive hemorrhages associated with the therapy. Additionally, fever has been seen with rGM-CSF administration and in several cases occurred while the patient was neutropenic. One patient developed hypotension as a possible side effect of rGM-CSF and was dose reduced to 50% without significant improvement in blood pressure. An additional patient developed staphylococcal endocarditis during the third cycle of therapy. Further chemotherapy could not be administered but she was found to have a partial remission after this third cycle. All patients who were treated with more than one cycle of therapy required carboplatin dose reductions. The patient who received three complete cycles of therapy required two 25% dose reductions and the second patient a 25% dose reduction following administration of cycle one.

SIGNIFICANCE

Although final interpretation of the results of this study will require enrollment of additional patients, several tentative conclusions can be drawn. First, GM-CSF as administered in this trial is incapable of preventing the neutropenia and thrombocytopenia expected after administration of high doses of carboplatin. rGM-CSF can cause fevers while patients are neutropenic. This side effect of GM-CSF will likely make it necessary to admit patients to the hospital to receive empiric therapy for fever in association with neutropenia. Although it may yet be possible that GM-CSF will reduce the incidence and/or severity of neutropenia and its associated complications, administration of GM-CSF following high doses of carboplatin will likely not reduce the need for hospitalization and administration of empiric antibiotic therapy. Finally, GM-CSF appears to have no effect upon the severity and duration of thrombocytopenia and the need for intensive platelet transfusion support. The preliminary results would suggest that an agent capable of enhancing recovery of both platelets and granulocytes will be necessary to allow safe exploration of high-dose carboplatin therapy.

PROPOSED COURSE

We will continue to accrue patients to this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09330-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Efficacy of Immunomodulating Doses of IFN Gamma in Metastatic Melanoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
Others:	S. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, NCI
	R. Steis	Medical Officer	CRB, NCI
	L. VanderMolen	Medical Staff Fellow	CRB, NCI
	K. Conlon	Senior Staff Fellow	CRB, NCI
	J. Janik	Expert	CRB, NCI
	R. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba, L. Hartmann); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recombinant interferon gamma has previously been shown to enhance several immunologic parameters in patients with malignant melanoma rendered disease-free with surgery. In a prior BRMP protocol, we demonstrated that there was an optimal immunomodulatory dose of interferon gamma that was lower than the maximally tolerated dose of this agent. The purpose of this protocol is to test three different doses of recombinant interferon gamma in patients with metastatic disease to see if there is an optimal immunomodulatory dose in these patients. After we accrue 5 patients at each dose level, we will determine whether there is a dose that is superior in terms of monocyte activation. We then plan to treat at least 12 additional patients at this dose to reliably determine a response rate to therapy.

To date we have enrolled 13 patients on this study. In general, therapy has been well tolerated and toxicities have been mild. One patient declined further therapy for personal reasons after 1 week of treatment. Another patient experienced a severe exacerbation of dermatomyositis requiring discontinuation of therapy. One patient at the 0.25 mg/sq m dose has had a mixed response to treatment. Two other patients had stable disease at the end of 2 months of therapy and continued treatment for a total of 4 months at which time progressive disease was demonstrated. The remainder of the 9 patients evaluable for tumor response had progressive disease. The effects of different doses of interferon gamma on the immune system have not yet been determined. We plan to finish enrollment onto the 3 different dose levels and then select the dose level that seems to be the best in terms of boosting the immune system and to treat at least 12 more patients at this dose level. We anticipate that this protocol can be completed within the next year.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, NCI
Ronald Steis	Medical Officer	CRB, NCI
Louis VanderMolen	Medical Staff Fellow	CRB, NCI
Kevin Conlon	Senior Staff Fellow	CRB, NCI
John Janik	Expert	CRB, NCI
Robert Fenton	Expert	CRB, NCI

OBJECTIVES

1. To explore the immunological effects and toxicity of different doses of recombinant interferon gamma in patients with metastatic malignant melanoma.

2. To record the antitumor effects of these interferon gamma regimens in patients with metastatic malignant melanoma.

3. To estimate the response rate to the most effective immunomodulatory regimen of recombinant interferon gamma determined from the first part of this study in patients with metastatic malignant melanoma.

METHODS EMPLOYED

Patients who met the following criteria are eligible:

Biopsy proven metastatic malignant melanoma, performance status 70% or greater on the Karnofsky scale, life expectancy of at least 3 months, bidimensionally measurable disease, no prior chemotherapy or immunotherapy, and adequate physiological function.

After initial clinical evaluation, patients are assigned to one of three groups. Each group receives a different dose of interferon gamma with the first group receiving 0.01 mg/sq m, the second 0.1 mg/sq m, and the third 0.25 mg/sq m. Patients receive interferon gamma by daily subcutaneous injection for a period of 2 months. During the course of treatment frequent blood sampling is performed to assess the effects of treatment on the immune system. After accruing 5 patients to each group, an analysis will be performed to determine which if any of the three doses optimally modulate the immune system. We will then accrue 12 more patients at that dose to determine the response rate to this regimen.

MAJOR FINDINGS

Thirteen patients have been entered onto this study. One patient refused to continue treatment after less than 1 week of therapy for personal reasons. Another patient was taken off study because of the development of severe

dermatomyositis felt to be exacerbated by treatment with interferon gamma. Two patients experienced Grade III neutropenia. All other toxicities were Grade II or less and consisted of myalgia, arthralgia, fatigue, nausea, vomiting, diarrhea, chills, fever, headache, and weight loss. One patient treated at the 0.25 mg/sq m dose had evidence of progressive disease at the end of 1 month of treatment and was taken off study. Thirty days later, the patient had evidence of a delayed response to treatment and is considered a mixed responder and has been restarted on the same dose of interferon gamma. Two patients had stable disease at the end of 2 months of treatment and continued therapy for a total of 4 months until there was evidence for progressive disease. All other patients had evidence of progressive disease at either one or two months. Two patients currently on treatment have not been evaluated for disease response. The immunological assays designed to assess monocyte activation will be performed after 5 patients have completed treatment in each of the 3 groups mentioned above.

SIGNIFICANCE

A variety of biological agents have antitumor efficacy in patients with malignant melanoma. The exact mechanism by which these agents bring about tumor responses is not known but studies in animal models suggest that tumor responses are produced by an indirect effect mediated by immunostimulatory effects of these agents. Prior studies using the maximally tolerated dose of interferon gamma have shown that this agent is capable of bringing about tumor responses in malignant melanoma. However, a prior study at the BRMP indicated that doses substantially lower than the maximally tolerated dose bring about equal or greater immunomodulatory effects than the maximally tolerated dose. That study however was performed in patients rendered disease free by surgery but at high risk for recurrence. In this study we are attempting to determine the optimal immunomodulatory dose in patients with measurable metastatic melanoma and to determine if this optimal immunomodulatory dose is capable of bringing about tumor responses. Interferon gamma is the first agent studied in detail for the determination of an optimal immunomodulatory dose. If greater antitumor effects are seen at the optimal immunomodulatory dose than have been reported with maximally tolerated doses of interferon gamma, this study will have enormous impact on immunotherapies as it would suggest that other immunomodulatory agents might be more active at doses lower than the maximally tolerated dose.

PROPOSED COURSE

After 4 more patients are accrued to this study, Part 1 will be completed and the immunological assays will be performed to determine the optimal immunomodulatory dose. If this dose turns out to be 0.25 mg/sq m, the study will be terminated because a Phase II study of interferon gamma in malignant melanoma patients with metastatic disease has been performed at that dose and had low activity. If the optimal immunomodulatory dose is either of the 2 lower doses, 12 more patients will be entered at that dose to determine the response rate to therapy. If no dose appears superior to the others we will treat 12 patients at 0.1 mg/sq m. If we observe at least 4 responses in the first 17 patients treated at a given dose level, we will go on to enroll a total of 37 patients at that dose level in order to reliably determine a response rate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09331-01 CRB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phase Ib Trial of Poly ICLC in Combination with IL-2 in Patients with Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. P. Creekmore	Chief	BRB, NCI
Others:	J. Janik	Expert	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. Fenton	Expert	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS (if any)

Frederick Memorial Hospital (C. Donovan); Program Resources, Inc., Frederick, Maryland 21701 (W. J. Urba, J. Beveridge); Cancer Therapy Evaluation Program (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIC-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This trial was designed to explore the immunomodulatory effects of poly ICLC in combination with IL-2, and to determine a dose combination with maximal immune augmentation consistent with tolerable toxicity. Poly ICLC is one of a family of polyribonucleotides originally studied in humans for the interferon-inducing and antiproliferative activities previously observed in animal studies. Poly ICLC is a prototypical biological response modifier which has such potent and reproducible immune and antitumor effects in animal models that it is routinely used as the positive control agent against which new immunomodulators and cytokines are compared. In prior clinical studies, no optimal immunomodulatory dose was determined and most studies have concentrated on determining maximum tolerated doses and doses which were capable of inducing interferon. In fact, no evidence has been seen for consistent antitumor effects at maximally tolerated doses. Experimental animal data, however, suggest that the maximum tolerated dose is neither the optimal tolerated dose nor the optimal immunotherapeutic dose, both of which are considerably lower than the MTD. In this study, poly ICLC doses below the MTD are explored to evaluate immunomodulatory effects and search for antitumor activity. Poly ICLC is given initially alone and subsequently in combination with IL-2. The IL-2 regimen employs twice weekly administration by 24-hour continuous infusion using a (moderate) dose of 3 million units/m2 which has previously been shown to generate circulating endogenous LAK activity and to be well tolerated for long periods of outpatient administration. Patients first receive poly ICLC intramuscularly as a single agent for one month. In subsequent 1-month cycles, poly ICLC is given IM b.i.w., together with IL-2 twice weekly. Cohorts of patients receive fixed poly ICLC doses below the known MTD to search for an optimum immunomodulatory dose.

PROJECT DESCRIPTION

PERSONNEL

Stephen P. Creekmore	Chief	BRB, NCI
John Janik	Expert	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Robert Fenton	Expert	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, NCI

OBJECTIVES

1. To explore the immunological effects and toxicity of Poly ICLC alone and in combination with IL-2.

2. To record the antitumor effects of the regimen.

METHODS EMPLOYED

Patients are required to have measurable tumor, good performance status (70% Karnofsky or greater), adequate physiological function (creatine < 1.5 mg/dL, bilirubin < 1.8 mg/dL, WBC > 3000/cmm, platelets > 100,000, EKG and stress test with only minor abnormalities, FEV-1 > 70% predicted, total calcium < 10.5, albumin > 3.0), expected survival greater than 3 months, and no brain metastases. Tumor types are restricted to histologically confirmed solid tumors in patients not eligible for surgery, standard chemotherapy or radiotherapy of known benefit in terms of prolongation of survival or palliation of symptoms.

Patients are treated with poly ICLC and IL-2 in the following regimen:

> First month: poly ICLC at the indicated dose by intramuscular injection once during the first week and then twice weekly for three weeks for a total of 7 doses (e.g., Tuesday/Friday schedule).
>
> Second month and later: IL-2 at 3 million units/m^2 by 24 hour iv infusion b.i.w. (outpatient), together with poly ICLC at the indicated dose by intramuscular injection twice weekly at the beginning of each IL-2 infusion.

Cohorts of 5 patients each receive fixed poly ICLC doses selected from the following dose levels: 1000, 300, 100, 30, 10 micrograms/meter squared.

Patients are assessed for response after 3 months. Responding patients continue on therapy until disease progression.

MAJOR FINDINGS

Twenty-three patients have been entered on the trial. Seventeen completed treatment with both Poly ICLC and IL-2 and are eligible for analysis for

toxicity and immunomodulatory effects. Four were removed from study for toxicity (3 for fatigue, 1 exacerbation of arthritis). No partial or complete responses have been seen. Analysis of immunomodulatory effects is underway but preliminary results show that poly ICLC alone had no effect on circulating natural killer and LAK cell activity and, when given in combination with IL-2, did not increase NK or LAK cell activity above that which would be expected with IL-2 alone. Analysis of the affects of poly ICLC on serum interferon and neopterin levels and induction of 2', 5' oligoadenylate synthetase activity are pending.

SIGNIFICANCE

This study was initiated because of the profound antitumor activity that poly ICLC has in a variety of animal tumor systems. In addition, previous studies with poly ICLC have not been designed in a fashion capable of fully exploring immunomodulatory effects of this agent but instead concentrated on determining the maximally tolerated dose. Although the laboratory analysis of the immune effects of poly ICLC in our patients has yet to be completed, it appears that poly ICLC doses below the maximally tolerated dose do not have significant effects on effector cell function. Other effects of poly ICLC on the immune system however need to be evaluated to determine its ultimate role in cancer therapy.

PROPOSED COURSE

The initial trial will be closed to further entry at the completion of the present dose level. A follow-on trial will be considered depending on completion of the laboratory evaluation of the immunomodulatory effects of these agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09332-01 CRB

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phase I Evaluation of Interleukin-1 Alpha

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
Others:	R. Steis	Medical Officer	CRB, NCI
	S. Creekmore	Chief	BRB, NCI
	D. Longo	Associate Director	OAD, NCI
	L. VanderMolen	Medical Staff Fellow	CRB, NCI
	J. Oppenheim	Chief	LMI, CRB

CONTINUED ON NEXT PAGE

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI (M. Sznol); Frederick Memorial Hospital, Frederick, MD (N. Wells)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interleukin-1 (IL-1) is a polypeptide produced by human cells that has pleiotropic biological effects and is involved in inflammation, immunological challenges, and the response to infection. By itself, or in conjunction with other agents, IL-1 has been demonstrated to activate T cells, B cells and large granular lymphocytes. It induces the production of colony stimulating factors, interleukins and interferons by cells in the immune system. IL-1 has direct anti-proliferative activity against certain human tumor cell lines in vitro. It has also demonstrated antitumor effects in vivo against several syngeneic murine tumors. Animal studies have also shown that IL-1 can protect and restore the bone marrow from injury due to chemotherapeutic agents or radiation. IL-1 could be potentially beneficial in the treatment of human cancer as a result of its direct anti-proliferative activity, its extensive immunoenhancing properties, or its bone marrow protective and restorative effects. We plan to begin the clinical investigation of this agent by performing a Phase I study that will determine the toxicity, the immunological effects, and the hematological effects of IL-1 alpha administered intravenously daily for 7 consecutive days. After we determine a maximally tolerated dose of IL-1 alpha, we will combine IL-1 alpha with indomethacin to determine if indomethacin ameliorates the toxicity or enhances the immunological or hematological effects of IL-1 alpha.

To date, three patients have received treatment at the lowest dose level (0.01 mcg/kg). Side effects included chills (requiring Demerol), fever, fatigue, and headache but no significant hypotension or pulmonary symptoms. At this dose level, no changes were noted in hematologic values or blood chemistries.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald Steis	Medical Officer	CRB, NCI
Stephen Creekmore	Chief	BRB, NCI
Dan Longo	Associate Director	OAD, NCI
Louis VanderMolen	Medical Staff Fellow	CRB, NCI
Joost Oppenheim	Chief	LMI, CRB
Kevin Conlon	Senior Staff Fellow	CRB, NCI
John Janik	Expert	CRB, NCI
Robert Fenton	Expert	CRB, NCI

OBJECTIVES

1. To determine the toxicity of and the maximally tolerated dose of IL-1 alpha given by the intravenous route once a day for 7 consecutive days.

2. To determine the immunological effects of IL-1 alpha given at these doses according to this schedule.

3. To determine the hematologic effects of IL-1 alpha given by this schedule.

4. To determine the effect of indomethacin on the toxicity and the immunological and hematological effects of IL-1 alpha.

5. To determine the pharmacokinetics of intravenously administered IL-1 alpha.

6. Note any antitumor effects of the agent.

METHODS EMPLOYED

Patients are eligible for this trial if they have a histologically confirmed solid tumor which is not curable by surgery, radiotherapy or standard chemotherapy. Patients must have failed to respond to standard treatment regimens or have a malignancy for which no effective treatment exists. They must have a Karnofsky performance status equal to or greater than 70%, a life expectancy of at least 3 months, evaluable or measurable disease, and adequate physiological function. After initial clinical evaluation, the patients will be admitted to the hospital and be treated with IL-1 alpha given by intravenous infusion over 15 minutes daily for 7 consecutive days. This is the Phase I IND protocol for this agent and patients will be treated at the following dose levels: 0.01 mcg/kg, 0.03 mcg/kg, 0.1 mcg/kg, 0.3 mcg/kg, 1 mcg/kg, 3 mcg/kg, 6 mcg/kg, and 10 mcg/kg. At least three patients will be entered at each dose level until a maximum tolerated dose has been defined. Once this has been defined for IL-1 alpha, additional patients will be treated with the combination of indomethacin 25 milligrams every 8 hours plus IL-1 alpha beginning at 2 dose levels below the maximum tolerated dose and escalating by the same schedule until the maximum tolerated dose with indomethacin has been determined.

MAJOR FINDINGS

This protocol has just recently been approved by the Food and Drug Administration, the Institutional Review Board of the Clinical Oncology Program of the NCI, and the Institutional Review Board of the FCRF. To date, three patients have received treatment at the lowest dose level (0.01 mcg/kg). Side effects included chills (requiring Demerol), fever, fatigue, and headache but no significant hypotension or pulmonary symptoms. At this dose level, no changes were noted in hematologic values or blood chemistries.

PROPOSED COURSE

We plan to treat patients as outlined above and expect to complete this Phase I evaluation of this agent within the next year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09333-01 CRB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phase I Trial of I.P. OVB3-PE in Ovarian Cancer Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
Others:	R. Steis	Medical Officer	CRB, NCI
	D. Longo	Associate Director	CRB, NCI
	S. Creekmore	Chief	BRB, NCI
	K. Conlon	Senior Staff Fellow	CRB, NCI
	L. VanderMolen	Medical Staff Fellow	CRB, NCI

CONTINUED ON NEXT PAGE

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (W. J. Urba, L. Hartmann); Cancer Therapy Evaluation Program (M. Sznol); Frederick Memorial Hospital, Frederick, MD (C. Main); Fox Chase Cancer Center, Philadelphia, PA (M. Bookman, R. Young, R. Ozols, T. Hamilton); Duke Univ. Medical Center, Durham, NC (A. Frankel, B. Gould)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, MD 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

OVB3-PE is an immunoconjugate comprised of a IgG2b murine monoclonal antibody that recognizes an antigen on the surface of all human ovarian cancers and pseudomonas exotoxin joined by means of a thioether bond. Pseudomonas exotoxin is a bacterial toxin that kills cells by inhibiting protein synthesis. This immunotoxin was shown to kill human ovarian cancer cells in vitro. Studies of this immunotoxin in mice with the OVCAR 3 asgites tumor demonstrated that mice receiving the immunotoxin had a significantly prolonged survival. We conducted a Phase I study of the OVB3-PE immunotoxin given as multiple intraperitoneal infusions in patients with refractory ovarian carcinoma involving the peritoneal cavity in order to determine the toxicity of treatment, to determine serum and intraperitoneal levels of OVB3-PE, to determine the types of host anti-immunotoxin antibodies that might develop, and to assess any antitumor activity of the immunotoxin. Seventeen patients were entered onto this nonrandomized, Phase I multi-dose escalation study. Patients were entered from several different cooperating institutions. The BRMP entered 3 patients - one at the 2 mcg/kg dose level and 2 at the 10 mcg/kg dose level. One of our 3 patients sustained grade 3 pain and neurological toxicity after treatment with 10 mcg/kg. Similar but not identical grade 3 neurological toxicity was also observed in another patient treated at the Medicine Branch, NCI and as a result, no further dose escalations occurred beyond 10 mcg/kg. Despite intensive investigation, the mechanism for the neurotoxicity in these patients has not been elucidated. The protocol has been amended to treat 4 more groups of patients all at 5 mcg/kg but with the number of doses increasing from 3 to 8, all administered within 12 days. Patients will begin treatment on the revised schedule starting in July 1989.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald Steis	Medical Officer	CRB, NCI
Dan Longo	Associate Director	CRB, NCI
Stephen Creekmore	Chief	BRB, NCI
Kevin Conlon	Senior Staff Fellow	CRB, NCI
Louis VanderMolen	Medical Staff Fellow	CRB, NCI
Robert Fenton	Expert	CRB, NCI
John Janik	Expert	CRB, NCI
Ira Pastan	Senior Investigator	DCBD, DCT, NCI
David Fitzgerald	Senior Investigator	DCBD, DCT, NCI
Mark Willingham	Senior Investigator	DCBD, DCT, NCI
Eddie Reed	Senior Investigator	MB, NCI

OBJECTIVES

1. Assess the toxicity of the immunotoxin OVB3-pseudomonas exotoxin (OVB3-PE) given as single or multiple intraperitoneal infusions in patients with refractory ovarian carcinoma limited to the peritoneal cavity.

2. Determination of intraperitoneal and serum pharmacokinetics of OVB3-PE.

3. Determine the time course of development of and characterize the types of anti-immunotoxin antibodies that develop.

4. Evaluate the delivery of immunotoxin to target neoplastic tissues and the mechanism(s) of tumor resistance including antigen modulation.

5. Determine, in a preliminary fashion, any antitumor effects of this therapy.

METHODS EMPLOYED

Patients are eligible for this trial if they have histological confirmation of refractory invasive epithelial carcinoma of the ovary involving the peritoneal cavity that can be serially evaluated by noninvasive or invasive techniques. Patients must have received treatment with a platinum based chemotherapy regimen at some point in the course of their disease. They must have a Karnofsky performance status of greater than 70% and a minimum life expectancy of 3 months. They must have adequate physiological function and have no significant levels of serum neutralizing antibodies to pseudomonas exotoxin. Patients who have previously received murine monoclonal antibodies are excluded.

This is a nonrandomized, Phase I, fixed, multi-dose escalation study. Patients who are eligible for treatment undergo Tenckhoff catheter placement and receive OVB3-PE intraperitoneally on days 1 and 4 at the following dose levels: 1, 2, 5 and 10 mcg/kg. Frequent blood and peritoneal fluid samples are obtained during

the course of treatment. Approximately 4 weeks after therapy, patients have restaging evaluations that frequently included peritoneoscopy.

MAJOR FINDINGS

This protocol is a multi-center study the overall results of which will appear in other intramural project reports. The BRMP treated 3 patients on this study with the following results. One patient received 2 doses of 2 mcg/kg OVB3-PE and had mild fever, increase in LDH, and abdominal pain as the only toxicities. One month after treatment she developed peritonitis related to her Tenckhoff catheter and required intravenous antibiotics. She had a transient, minor reduction in the size of a retroperitoneal lymph node that had been biopsy proven prior to treatment to be malignant. Her serum did not neutralize OBV3-PE at day 14 but it did on day 34. The other 2 patients both received 10 mcg/kg of OVB3-PE. One patient sustained grade 3 abdominal pain and grade 3 neurological toxicity manifesting as a diffuse encephalopathy. She also experienced moderate nausea and vomiting as well as transient increases in her liver function studies. When she was re-evaluated post treatment, a peritoneoscopy was attempted; however, her bowel was perforated and she required a laparotomy. There was evidence of prior serosal inflammation and fibrosis with dense adhesions thought to be related to her prior treatment. She clinically had evidence of progressive disease. Because of the bowel perforation, multiple biopsies were not performed. The 3 biopsies which were taken were negative for tumor. CT of the abdomen and pelvis also showed 2 new masses that could not be biopsied at laparotomy. This patient had OVB3-PE neutralizing antibodies in her serum on day 14. The second patient treated at the 10 mcg/kg dose had no toxicity from the treatment and had evidence of progressive disease at re-evaluation.

Because grade 3 neurotoxicity was also seen in a patient treated at the Medicine Branch at the 10 mcg/kg dose, it was decided to stop the dose escalation and enroll more patients at the 5 mcg/kg dose. Two more patients were entered at this dose at another center and had no significant toxicity.

Both patients who had significant neurological toxicity had undetectable serum levels of OVB3-PE. Cerebral spinal fluid also had no detectable OVB3-PE. Because of the clinical findings, OVB3 itself and OVB3-PE were tested for reactivity with a variety of human brain tissues and there was no evidence of binding with any brain tissue or vascular tissue in the brain. The mechanism of the neurotoxicity observed in this trial is unknown.

SIGNIFICANCE

This study shows that up to a dose of 5 micrograms/kg the OVB3-PE immuno-conjugate can be safely administered intraperitoneally to patients with ovarian cancer. The one minor regression in a retroperitoneal lymph node that was observed is encouraging and suggests that further evaluation of this immunoconjugate is warranted. The frequent development of anti-immunoconjugate antibodies both in the serum and peritoneal fluid suggests that if this therapy is to be delivered in an effective manner, treatment must be completed in less than two weeks. The proposed alterations in the study reflect this line of reasoning.

PROPOSED COURSE

This study has been amended in the following manner. Patients will receive 5 mcg/kg of OVB3-PE on days 1, 4 and 7 in the first group, on days 1, 3, 5, and 7 in the second group, on days 1, 2, 4, 5, 7 and 8 in the third group, and on days 1, 2, 3, 4, 5, 6, 7, 8 in the fourth group. Patients will be entered in groups of 3 until the maximum tolerated dose is determined. Patients will be entered onto this amended schedule starting in July 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09334-01 CRB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phase I Trial of Anti-CD3 Monoclonal Antibodies in Patients with Advanced Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	L. VanderMolen	Medical Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS (if any)

Medicine Branch, NCI (J. Ashwell); Cancer Therapy Evaluation Program, NCI (M. Sznol); Program Resources, Inc., Frederick, MD (W. J. Urba, S. Markus); Frederick Memorial Hospital, Frederick, MD (T. Watson)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We undertook a Phase I study of anti-CD3 monoclonal antibodies in patients with solid tumors to determine the immunomodulatory properties of this antibody at various doses and to determine its antitumor effects. The protocol was designed to treat five patients at one of six dose levels including 1, 10, 30, 100, 300, and 1,000 micrograms per patient. Patients were to be treated by a three-hour intravenous infusion, four doses over two weeks administered three days apart. Toxic effects included fever, chills, mild nausea and vomiting, and severe headache in patients at the 30 and 100 microgram dose levels. The dose-limiting toxicity was severe headache and the maximum tolerated dose of anti-CD3 administered by three-hour infusion was 30 micrograms. A syndrome of aseptic meningitis developed in one patient at the 30 microgram dose level and all three patients at the 100 microgram dose level. Cerebrospinal fluid showed an abnormal lymphocytosis with an elevated protein in patients who had developed severe headache with photophobia and stiff neck. No antitumor responses were seen. The immunologic effects induced by antibody administration are currently being examined.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Louis VanderMolen	Medical Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. To determine the toxicity of multiple low doses of anti-CD3 antibodies in patients with advanced cancer.

2. To determine the immunomodulatory properties of different doses of anti-CD3 antibodies in patients with advanced cancer.

3. To record any tumor responses seen in patients with advanced cancer after treatment with multiple doses of anti-CD3 antibodies.

METHODS EMPLOYED

Patients to be considered for this study had a histologically confirmed diagnosis of a solid tumor for which there was no effective therapy or for which effective therapies were previously tried and failed. Patients with a Karnofsky performance status of 70 or greater were eligible and they must have had adequate pulmonary, renal, and hepatic function. Patients must not have received any form of therapy within the four weeks prior to entry and patients with controlled, treated central nervous system metastasis were eligible.

Consecutive groups of five patients were treated at a single dose level of anti-CD3. The dose levels tested were 1, 10, 30, and 100 micrograms per patient. All doses of monoclonal antibody were administered in the outpatient clinic by three hour infusion. Patients received four doses given three days apart. Serial blood specimens were obtained prior to and during treatment to assess the number and state of activation of peripheral blood cells. In addition, sera were collected to measure induction of lymphokines. Selected patients with available tumor had serial tumor biopsies performed to examine effects of treatment on tumor histology.

MAJOR FINDINGS

A total of 18 patients have been entered on this study and all are evaluable for toxicity. Fifteen of the 18 patients are evaluable for response. One patient experienced marked tumor progression after only three doses and did not receive the fourth dose. One patient received only three doses of anti-CD3 because of the development of severe headache; he also developed tumor progression. One patient received only two doses of antibody and two patients each received only one dose of antibody and were removed from study because of severe headache at the 100 microgram dose level.

Five patients were treated at the 1 microgram dose level and five patients were treated at the 10 microgram dose level; all ten patients received all four doses of treatment. Five patients received 30 micrograms per dose with four receiving all four doses of antibody and one patient receiving only three doses of antibody. Three patients were enrolled at the 100 microgram dose, one patient received two doses and two patients received one dose each. The toxicities observed were dose-related. Patients receiving one microgram of antibody experienced no significant toxicities. Patients receiving 10 micrograms of antibody experienced mild fever with occasional associated chills with some very mild nausea and no vomiting. Symptoms tended to resolve within hours of completion of the antibody infusion. There were a few patients with mild headaches. The 30 microgram dose was associated with mild headaches in most patients easily controlled with acetaminophen but severe headache prompted the performance of a spinal tap in one patient. This patient was found to have an abnormal number of lymphocytes in the spinal fluid and an elevated protein. All cultures were negative and the patient's symptoms resolved spontaneously. At 100 micrograms, all three patients developed severe Grade IV headaches preventing further treatment. All three patients also had fever and chills and two of three patients developed a stiff neck in association with their headache. All three patients underwent an examination of the cerebrospinal fluid. Abnormal numbers of lymphocytes were found in two of the three patients and mild to moderate elevation of the colony stimulating factor protein was found in all three. No bacterial or fungal organisms were cultured from the spinal fluid. Among the 15 patients evaluable for response, there were 4 patients with stable disease and 11 patients with progressive disease. There were no partial or complete responders.

Immunomodulatory effects are currently being examined.

SIGNIFICANCE

Anti-CD3 has great potential as a biological response modifying agent in the treatment of cancer. Its major use will probably be in combination with other biologic agents. In order to know what dose of this agent to employ, it was important to do a Phase I study. The maximum tolerated dose in our study in patients receiving anti-CD3 by three hour infusion and in patients with relatively normal immune systems, not compromised by treatment with azathioprine or steroids as is the case in renal transplant patients, reveals a maximum tolerated dose (30 mcg) 1/50 that used on a regular basis in treatment of renal transplant allograft rejection.

PROPOSED COURSE

We are currently treating five additional patients at the maximum tolerated dose to assess efficacy and further delineate toxic effects. We plan to initiate further study of this agent at doses of 30 micrograms and above given by bolus infusion to see if this changes the toxicity experienced.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09335-01 CRB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

MoAb (Anti-T3) Treatment of Patients With Lymphoproliferative Disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	J. W. Clark	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	K. Conlon	Senior Staff Fellow	CRB, NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (T. Watson); Medicine Branch, NCI (J. Ashwell)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a Phase I study of a human monoclonal antibody to the CD3 antigen, an antigen that is associated with the T-cell receptor. We examined patients for evidence of tumor response to monoclonal antibody treatment and also for immunomodulatory properties of the antibody. Anti-CD3 was to be given in a dose escalation manner with six groups of three patients each being treated with increasing doses. Only two patients have been treated on this protocol and each patient received 1 microgram of anti-CD3. There was no toxicity observed in either patient and neither patient exhibited a tumor response to monoclonal antibody treatment. There were no significant immunologic changes noted in the patients' peripheral blood during treatment.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Jeffrey W. Clark	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Kevin Conlon	Senior Staff Fellow	CRB, NCI

OBJECTIVES

1. To determine the toxicity of multiple low doses of anti-CD3 in patients with CD3-bearing lymphoproliferative disorders.

2. To determine the immunomodulatory effects of different doses of anti-CD3 antibodies.

3. To evaluate the clinical response of patients with CD3-bearing lymphoproliferative disorders to multiple doses of anti-CD3 antibodies.

4. To monitor patients for formation of anti-murine antibodies.

METHODS EMPLOYED

Patients to be considered for this study must have had a histologically and immunologically confirmed diagnosis of a CD3-bearing leukemia/lymphoma/ lymphoproliferative disorder confirmed by NIH Pathology review. We accept only patients with a Karnofsky performance status of greater than 60 with a life expectancy of at least four months. The patients must have had adequate pulmonary, renal and hepatic function and must not have received treatment with any other therapy in the four weeks prior to entry. Patients with treated and adequately controlled brain metastases were eligible. Consecutive groups of three patients each will be treated with doses of anti-CD3 of either 1, 10, 30, 100, 300, or 1,000 micrograms. Only two patients have been treated on this protocol so far and both patients were treated at the 1 microgram dose level. Serial blood specimens were obtained prior to and during treatment to assess the state of activation of peripheral blood cells and their numbers.

MAJOR FINDINGS

A total of two patients were entered on this study and both are evaluable for toxicity and response. Both patients had mycosis fungoides. Both patients received all ten doses of therapy. No toxic effects were observed during the administration of the monoclonal antibodies in either patient. Both patients did experience progressive disease during treatment and went on to treatment with other agents.

Immunologic changes have not been detected on the cell surface of peripheral blood lymphocytes obtained during treatment at the 1 microgram dose.

SIGNIFICANCE

The significance of this study is in the potential use of anti-CD3 as a direct growth inhibiting monoclonal antibody for malignant T cells. The other possible significance is for the use of anti-CD3 to activate the patient's immune system, particularly tumor specific T cells, to participate in an antitumor response. The trial is incomplete at this time and no specific conclusions can be drawn.

PROPOSED COURSE

This study is currently open and we are actively recruiting patients with CD3 bearing malignancies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09336-01 CRB

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Short-Course ProMACE-CytaBOM for Stages II-IV Diffuse Aggressive Lymphoma

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD; Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study was designed as a pilot study to test the feasibility and efficacy of administering a dose-intense version of a standard combination chemotherapy regimen ProMACE-CytaBOM for patients with Stages II, III, and IV diffuse aggressive lymphoma. The treatment consists of six 2-week cycles, for a total of twelve weeks. To date, there have been eighteen evaluable patients entered on study: fifteen have achieved a complete response (83%). There have been 2 relapses (14%), both following short complete remissions. In conclusion, these preliminary results indicate that the treatment can be safely given, though some patients require dose-reductions. Early response rates are equivalent to standard ProMACE-CytaBOM.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI

OBJECTIVES

1. To determine the feasibility of administering a dose-intense ProMACE-CytaBOM to patients with Stages II, III and IV diffuse aggressive lymphoma in a pilot study, 12-week outpatient treatment regimen.

2. To determine the complete response rate, disease-free survival and overall survival of previously untreated patients with advanced stages of diffuse lymphoma treated with short-course ProMACE-CytaBOM.

3. To compare the results with those of age, stage, and sex-matched patients treated with standard ProMACE-CytaBOM.

4. To calculate the dose-intensity of drugs actually delivered with short-course ProMACE-CytaBOM and compare it to the actual dose-intensity of drugs received with the standard ProMACE-CytaBOM.

5. To determine whether this regimen demonstrates sufficient efficacy to warrant a recommendation for its inclusion in a large-scale randomized trial.

METHODS EMPLOYED

Previously untreated patients, with a histologic diagnosis of diffuse aggressive lymphoma, [diffuse large cell (DHL), follicular large cell (NHL), and diffuse mixed (DML)], Stage II, III or IV, are eligible for treatment on this study. Patients must be > 15 years of age. There is no upper age limit. Patients will undergo complete staging work-up including CXR, thoracic and abdominal CT scan, lymphangiogram, liver/spleen scan, gallium scan, bone scan, bilateral bone marrow aspirates and biopsies, and liver biopsy. Other tests will be performed as indicated. Patients will receive the pilot regimen Short-course ProMACE-CytaBOM for a minimum of six two-week cycles for a minimum total duration of 12 weeks of treatment. ProMACE drugs (cytoxan, adriamycin, and etoposide) are given on day 1 of each cycle. CytaBOM drugs (cytarabine, bleomycin, oncovin, and methotrexate) are given on day 8. Prednisone is given daily in 2 week blocks alternating with one week off. Each cycle is 2 weeks long. Following cycle 4, the patient is completely restaged. All tests that were positive initially are repeated. If the patient is in complete remission, 2 more cycles of therapy are given. Otherwise, patients are treated for two cycles beyond the cycle in which a CR is documented. At the completion of therapy, the patient is followed at monthly intervals for the first six months, every other month for six months, every 3 months for a year, twice yearly for a year, and yearly thereafter. Bactrim is given for all patients throughout the entire treatment as prophylaxis against Pneumocystis carinii pneumonia.

MAJOR FINDINGS

Twenty-one patients have been entered on this study. Three patients are inevaluable: 2 are too early to evaluate. One 74 year old patient died suddenly at home in cycle 3, with normal blood counts. Coroner's report stated presumed arrhythmia (though no cardiac history). No autopsy was performed. Patient is evaluable for survival, but not for response. Of the 18 evaluable patients, 15 have achieved a complete response (83%). One patient required the addition of local radiation therapy to achieve CR. Two patients have relapsed (14%), both following short complete responses each lasting less than 4 months. One of the relapsed patients underwent autologous bone marrow transplant and is in second CR at 11+ months. The other relapsed patient is currently receiving further treatment. Four patients have died on this study. Two are induction failures who never achieved a complete response. One patient died in CR with bacterial pneumonia for which she refused any treatment (a reversible complication of therapy). One patient died suddenly during treatment. There have been no unexpected toxicities.

SIGNIFICANCE

Although the results of this regimen are quite preliminary with small numbers of patients and relatively short follow-up time, the response rate and relapse rate are equivalent to standard ProMACE-CytaBOM. Additional accrual and longer follow-up will enable firmer conclusions regarding the efficacy of this regimen.

PROPOSED COURSE

This study remains open and continues to accrue patients.

PUBLICATIONS

There have been no publications regarding this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09337-01 CRB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dose-Intense MOPP for Patients With Poor Prognosis Hodgkin's Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, NCI

COOPERATING UNITS (if any)

Radiation Oncology Branch, NCI (E. Glatstein); Laboratory of Pathology, NCI (E. S. Jaffe); Program Resources, Inc., Frederick, MD; Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This protocol was designed to treat the subset of Hodgkin's disease patients who are at highest risk for treatment failure.

From the previous study of advanced Hodgkin's disease, we have identified patients with Stage IIIB, IVB, IV (marrow) and massive mediastinal Hodgkin's disease as having lower response and survival rates. In order to improve the efficacy of MOPP therapy in this group of patients, dose-intensity of all drugs has been increased and GM-CSF is given following chemotherapy in an attempt to amelioratae the expected severe myelotoxic effects of these increased drug doses. To date, only two patients have been enrolled in the study and both are too early to evaluate.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI

OBJECTIVES

1. To evaluate the complete response rate, disease-free survival and the overall survival of patients with Hodgkin's disease treated with Dose-intense MOPP.

2. To calculate the dose-intensity of drugs actually delivered with DIMOPP and compare it to the dose-intensity of MOPP.

3. To assess the toxicity of this regimen and determine the benefit of GM-CSF in ameliorating the myelotoxicity of MOPP.

METHODS EMPLOYED

Previously untreated patients, at least 14 years of age, with a histologically documented diagnosis of Stage IIIB, IVB, IVA (marrow) and massive mediastinal Hodgkin's disease are eligible for this study. Patients undergo standard staging including chest x-ray, CT scans of chest and abdomen, lymphangiogram, gallium scan, liver/spleen scan, bone marrow biopsies, and liver biopsy. Patients with massive mediastinal disease will be seen in the Radiation Oncology Branch for simulation prior to beginning chemotherapy because these patients will receive radiation therapy following DIMOPP. At the completion of staging, all patients are treated with dose-intense MOPP consisting of nitrogen mustard 7.2 mg/M2 d.1 and 8; oncovin 1.4mg/M2 days 1 and 8; procarbazine 200mg/M2 P.O. days 1-8; prednisone 80mg/M2 P.O. days 1-8. Recombinant human granulocyte macrophage colony stimulating factor 10mcg/kg S.C. b.i.d. days 9-15. Patients receive a minimum of 6 cycles and 2 cycles beyond a complete response. When those patients diagnosed with massive mediastinal disease have achieved a complete response, radiation (10Gy to the original extent of the mass and an additional 20-25Gy to any residual mass following chemotherapy) will be delivered.

MAJOR FINDINGS

Two patients have been entered on study. Both continue on treatment and are too early to evaluate. No unexpected toxicities have been observed. Despite the GM-CSF, some dose reductions have been necessary.

SIGNIFICANCE

Not applicable.

PROPOSED COURSE

The study remains open to patients accrual. We plan to accrue at least 35 patients to determine the feasibility of administering this regimen and for assessing the response to this treatment for this group of poor prognosis patients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09338-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
MOPP/ABVD Followed by Radiotherapy for Massive Mediastinal Hodgkin's Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	P. L. Duffey	Research Specialist	OAD, NCI

COOPERATING UNITS (if any)
Radiation Oncology Branch, NCI (E. Glatstein)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)
- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with massive mediastinal Hodgkin's disease are known to have a somewhat poorer response to treatment than other Hodgkin's patients. This trial was designed to improve the response rate and disease-free survival of patients with any stage massive mediastinal Hodgkin's disease. Patients on this study are fully staged and simulated for radiation therapy prior to beginning chemotherapy, which consists of alternating cycles of two non-cross-resistant regimens, MOPP and ABVD for a total of six months of therapy. Patients who are in complete remission following chemotherapy receive radiation therapy delivered in a novel way. The first 10Gy are administered to the original extent of disease and an additional 35-40Gy is delivered to any residual mediastinal mass. To date, there are 65 evaluable patients, 55 (85%) achieved a complete response. There have been 10 relapses (18%). Thirteen patients have died, one with a treatment related pneumonitis. These results are the best that have been obtained in this subgroup of patients with Hodgkin's disease.

PHS 6219 (Rev. 1/84)

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI

OBJECTIVE

1. To determine the complete response rate, disease-free survival and overall survival of patients with massive mediastinal Hodgkin's disease of any stage treated with MOPP alternating with ABVD followed by radiotherapy to the mediastinum.

2. To examine the acute and the long-term toxicities associated with combined modality therapy delivered in this way.

METHODS

Previously untreated patients, 14 years of age or greater, with histologically documented Hodgkin's disease whose mediastinal mass is greater than one third of the largest chest diameter on a PA chest x-ray are eligible for this study. Patients with any stage disease are eligible. Patients undergo staging evaluation with CXR, CT scan of chest and abdomen, lymphangiogram, liver/spleen scan, gallium scan, bone scan, bilateral bone marrow aspirates and biopsies, and liver biopsy. Prior to treatment, all patients are seen in the Radiation Oncology Branch for simulation. Patients are then treated with monthly cycles of MOPP alternating with ABVD chemotherapy for a minimum of six months of therapy. MOPP treatment consists of nitrogen mustard 6mg/M2 iv days 1, 8; oncovin 1.4mg.M2 iv days 1, 8; procarbazine 100 mg/M2 po days 1-14; prednisone 40 mg/m2 po days 1-14. No therapy is given days 15-28. Each cycle is 28 days. ABVD consists of adriamycin 25mg/M2 iv days 29, 43; bleomycin 10 units/M2 iv days 29, 43; vinblastine 6mg/M2 iv days 29, 43; DTIC 375mg/M2 iv days 29, 43. No therapy is given days 44-56. Patients are restaged following month 4 (after 2 cycles of each regimen). Patients who are in complete remission receive two additional months of treatment before receiving radiation. Patients who continue to have a responding mediational mass continue to receive chemotherapy until the mass has remained stable for two months and are considered in clinical complete remission at that point. When blood counts have returned to normal following chemotherapy, radiation therapy begins. The initial 10Gy is delivered to the original extent of the mediastinal mass. An additional 35-40 Gy is administered to any residual mediastinal mass. Patients who achieve a complete remission are followed at monthly intervals for six months, every other month for six months, quarterly for a year and yearly thereafter. Patients who do not achieve a complete remission or who have progressive disease will not receive radiation therapy. They are eligible for treatment on the autologous bone marrow transplant protocol.

MAJOR FINDINGS

A total of 72 patients have been entered on study. Seven are not evaluable: three are too early and 4 were noncompliant and did not finish therapy. Of the 65 evaluable patients, 55 (85%) achieved a complete response. There have been 10 relapses (18%). Thirteen patients have died: one patient died of treatment-related pneumonitis, with no evidence of Hodgkin's disease. We then amended the protocol and reduced the total dose of radiation. Since the amendment, there have been no serious episodes of treatment-related pulmonary fibrosis. Only three patients have developed infiltrates on CXR and two required steroid treatment.

Of the ten relapsed patients: 6 patients received MOPP reinduction and all achieved a second complete remission: four of the six continue in second CR at 2+, 6+, 38+ and 44+ months; one of the six patients died of leukemia with no evidence of Hodgkin's disease; one patient relapsed from CR at two months and died of progressive disease. Of the other four relapses, 2 underwent high-dose chemotherapy and autologous bone marrow transplant, achieved complete response and continue free of disease; one patient was treated with LACE and died of progressive disease; one patient refused further therapy. In total, of the ten relapsed patients, 6 are alive and free of disease.

SIGNIFICANCE

These results are the best that have been obtained in patients with massive mediastinal Hodgkin's disease. The results are particularly impressive because our group of patients also included patients with advanced stages of disease. This data also demonstrates that the bulk of the mass is a more important prognostic factor than stage in patients with Hodgkin's disease.

PROPOSED COURSE

The protocol is closed to further accrual. The replacement protocol is BRMP 8901, Dose-intense MOPP for Stage IIIB, IVB, IVA (MARROW+) and Massive Mediastinal Hodgkin's Disease which has begun to accrue patients.

PUBLICATIONS

Longo DL, Glatstein E, Young RC, Duffey PL, Hubbard SM, Phares J, DeVita VT Jr. Combined modality therapy for stage II, III, or IV massive mediastinal Hodgkin's disease: the NCI experience. Proc Am Soc Clin Oncol 1988;7:874.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09339-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
MOPP vs Radiotherapy for Early Stage Hodgkin's Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	P. L. Duffey	Research Specialist	OAD, NCI
	BRMP Staff		BRMP, NCI

COOPERATING UNITS (if any)
Radiation Oncology Branch, NCI (E. Glatstein); Laboratory of Pathology, NCI (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study was designed to compare the response rates and toxicities of MOPP chemotherapy vs radiation in the management of previously untreated laparotomy-documented patients with early stage Hodgkin's disease. Of the 51 evaluable patients randomized to MOPP, 50 (98%) achieved a complete response; 5 relapsed. Forty-nine of fifty-one randomized radiation-treated patients (96%) achieved a complete response: 16 patients (33%) relapsed. Four MOPP-treated patients (10%) and ten (20%) radiation-treated patients have died.

PHS 6348 (Rev. 1/84)

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI
BRMP Staff		BRMP, NCI

OBJECTIVES

1. To compare the complete response rate, disease-free survival and overall survival of patients with laparotomy-documented stage I or II Hodgkin's disease, randomized to treatment with either MOPP chemotherapy or radiation therapy.

2. To compare the acute and long-term toxicities resulting from each of these treatments.

METHODS EMPLOYED

Previously untreated patients, at least 14 years of age, with histologically-documented Hodgkin's disease will be staged completely including laparotomy. Patients with peripheral IA disease will be nonrandomly assigned to treatment with involved-field radiation. All other early Stage (IA central, IB and Stage II, excluding massive mediastinal) patients will be eligible for this study. Eligible patients are randomized between MOPP chemotherapy and subtotal nodal irradiation. Patients treated with MOPP receive a minimum of six cycles and two cycles beyond a complete remission: nitrogen mustard 6mg/M2 ivp days 1,8, vincristine 1.4mg/M2 ivp days 1,8, procarbazine 100mg/M2 po days 1-14 and prednisone 40mg/M2 po days 1-14. Patients randomized to radiation receive 40-45Gy radiation. Those who achieve a complete response are followed bimonthly for 6 months, every three months for a year, twice yearly for a year and yearly thereafter. Patients who relapse from complete response cross-over to the other arm of treatment if possible. (If a patient who relapses from a MOPP CR relapses with disseminated disease for which RT would not be appropriate, combination chemotherapy will be given). Patients whose initial CR is longer that one year are reinduced with MOPP. Those with initial CR's less than a year or who never achieved a CR would be eligible for the autologous bone marrow transplant protocol.

MAJOR FINDINGS

Randomized to radiation: A total of 51 patients have been randomized to radiation; 49 (96%) have achieved a complete response. Sixteen patients (33%) have relapsed from CR. Of the 16 relapses, 15 were reinduced with MOPP: 1 is too early, 11 achieved a second CR and 7 patients continue in CR (12-99+mos). One patient relapsed at 7 months and died with progressive disease. One relapsed and was treated with ABMT and is in 3rd complete response. Two patients died during MOPP reinduction with no evidence of disease. Three patients who did not achieve a second CR died with progressive disease. There

have been a total of 10 deaths (20%): 6 with progressive disease and 4 with no evidence of disease (2 sepsis during MOPP reinduction, 1 AIDS, 1 pneumonia).

Randomized to MOPP:

Of the 54 patients randomized to MOPP, 51 have completed therapy and are evaluable. Fifty (98%) achieved a complete response. Five patients have relapsed (10%). Of the five relapses, 2 patients crossed-over to radiation and both achieved a complete response: 1 continues in CR at 50 + months and 1 relapsed from RT at 6 months, received ABVD and died of pulmonary complications and pneumonia, without evidence of Hodgkin's disease. The three other relapsed patients (at 32, 20, and 2 months) had visceral disease and were reinduced with MOPP. One achieved a CR and remains in CR at 60+ mos. One patient died with progressive disease and sepsis. The third patient did not tolerate therapy and is alive with Hodgkin's disease. Overall on this arm, there have been 4 deaths: 1 relapse with progressive disease and 3 with no evidence of disease (1 MI, 1 sepsis, Pneumocyctis carinii pneumonia during initial induction, 1 treatment-related pulmonary fibrosis and pneumonia).

Nonrandomized to radiation:

Of the 42 patients on this portion of the study, 33 are Stage IA peripheral and 9 patients are miscellaneous (not randomized for various reasons). All 42 patients achieved a complete response. None of the stage IA patients have relapsed; two of the miscellaneous have relapsed. Both achieved a second CR with MOPP but both developed acute myelogenous leukemia. One patient died with leukemia and no Hodgkin's disease was present at death. The other patient was treated with aggressive therapy and has achieved a complete response lasting 6+ months.

SIGNIFICANCE

For the two randomized arms of the study, the complete response rates are equivalent. However there are fewer relapses with the MOPP treatment. The disease-free survival is significantly different with a p2 value of .004. Many of the relapsed patients are salvaged with other treatment and do achieve second complete remissions. The difference in overall survival between the two arms is statisticallly significant (p2=0.051). We continue to collect toxicity data, particularly the effects of therapy on fertility and on the incidence of second malignancies.

PROPOSED COURSE

The study remains open for accrual of patients. We continue to follow patients on study and assess long-term side-effects of each therapy.

PUBLICATIONS

Longo DL, Glatstein E, Young RC, Duffey PL, Hubbard S, DeVita VT Jr. Randomized trial of MOPP chemotherapy vs subtotal nodal radiation therapy in patients with laparotomy-documented early stage Hodgkin's disease. Proc Am Soc Clin Oncol 1987;6:812.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09340-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Observation Vs. Intensive Chemotherapy for Indolent Lymphoma

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	P. L. Duffey	Research Specialist	OAD, NCI

COOPERATING UNITS (if any)

Radiation Oncology Branch, NCI (E. Glatstein), Laboratory of Pathology, NCI (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study was designed ten years ago to compare the efficacy and the long-term survival of two very different therapeutic approaches towards previously untreated patients with indolent lymphoma: observation or delayed therapy (watch and wait) vs. initial intensive combination chemotherapy with ProMACE/MOPP flexitherapy to complete remission, followed by consolidative modified total nodal irradiation. To date there are no significant differences in disease-free survival or overall survival, although there are differences in complete response rates between the two randomized groups with 75% of patients randomized to intensive therapy achieving a complete response compared to a CR rate of 36% in those treated after a period of observation. The median follow-up time is six years, shorter than the median survival (10 years) of patients with these diagnoses. Continued follow-up may show a benefit for a subset of patients.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI

OBJECTIVES

1. To compare initial observation (Watch and Wait, WW) including palliative radiation therapy vs. initial intensive chemotherapy with ProMACE/MOPP flexitherapy followed by modified total lymphoid irradiation in previously untreated patients with advanced-stage indolent lymphoma with respect to response rate, disease-free survival and overall survival.

2. To evaluate the efficacy of modified total lymphoid irradiation following complete remission in decreasing the number of relapses or to alter the pattern of relapse.

3. To compare the quality of life of patients who have achieved a complete response compared to patients who remain on watch and wait.

METHODS EMPLOYED

Previously untreated patients, Stage III or IV with a histologically documented diagnosis of indolent lymphoma [follicular small cleaved cell (NPDL), follicular mixed cell (NML), diffuse well-differentiated lymphocytic (DWDL), diffuse small cleaved cell (DPDL-SC) and diffuse intermediately differentiated lymphocytic (DIDL)] are eligible for entry on this study. Following complete staging work-up including appropriate x-rays, scans, bone marrow biopsies and liver biopsy, patients are randomized to either watch and wait or initial intensive therapy. Patients who are not suitable for randomization due to B symptoms or other disease-related problems that require immediate systemic treatment are treated on the non-randomized intensive therapy arm. Patients on WW arm may receive up to 3 distinct fields of localized radiation therapy while continuing on WW. A patient on WW who develops either B symptoms, low blood counts due to marrow involvement, histologic progression, or progressive disease in a site that cannot be radiated, then crosses over to intensive treatment. Intensive treatment, whether for randomized patients, non-randomized or WW cross-over patients is ProMACE/MOPP flexitherapy. ProMACE chemotherapy is given for either 2 or 3 cycles depending on the rate of response to treatment. When the rate of disease regression slows, MOPP chemotherapy is administered for the same number of cycles as ProMACE had been administered. Following MOPP, ProMACE is again repeated. Thus patients receive either (2-2-2) or (3-3-3) cycles of chemotherapy. Patients who achieve a complete remission receive modified total lymphoid irradiation. Patients who do not achieve CR cross over to WW and continue to be observed until disease progression requires treatment. All patients are followed for survival on this study.

MAJOR FINDINGS

There have been a total of 137 patients entered on study with 119 randomized. Seven patients are not evaluable because each did not receive the treatment to which they were randomized: 3 on WW and 4 on Intensive therapy. Median follow-up is 6 years.

Randomized to Intensive Therapy: Of the 59 evaluable patients on this arm, 44 achieved a CR (75%). There have been 14 relapses (32%) and 15 (25%) have died. Twenty-eight patients (47%) continue alive and free of disease on this arm. There have been no unexpected toxicities with this therapy.

Randomized to Watch and Wait: There are 52 evaluable patients on this arm. Twenty-three patients remain on WW and are alive with lymphoma. Twenty-nine patients (56%) have crossed-over to intensive therapy. Four are too early to evaluate. 9/25 (36%) patients have achieved a complete response and 4 (44%) have relapsed from CR. Eleven patients (21%) have died. Five patients on this arm are alive and free of disease. Toxicities for patients who crossed-over are similar to those on the intensive therapy arm.

Non-randomized to Intensive Therapy: Of the 16 patients who were ineligible for randomization, 10 (63%) achieved a complete response. Three (33%) have relapsed and 8 have died. Four patients remain alive and free of lymphoma.

SIGNIFICANCE

The difference in complete response rate between the two randomized treatments is significantly different, however the overall survival is not. The median follow-up time of six years, however, does not allow for most of the patients to have reached the median survival expected with this disease.

PROPOSED COURSE

The study remains open to further patient accrual. Longer follow-up is necessary to determine if there is a survival difference between the different treatment approaches.

PUBLICATIONS

Young RC, Longo DL, Glatstein E, Ihde DC, Jaffe ES, DeVita VT Jr. The treatment of indolent lymphomas: watchful waiting v aggressive combined modality treatment. Sem in Hematol 1988;25:11-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09341-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
ProMACE-CytaBOM Treatment For Angiocentric Immunoproliferative Lesions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, NCI

COOPERATING UNITS (if any)
Institute of Allergy and Infectious Diseases, Bethesda, MD (A. S. Fauci); Laboratory of Pathology, NCI (E. S. Jaffe); Program Resources, Inc., Frederick, MD; Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)
- [X] (a) Human subjects
- [ ] (a1) Minors
- [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This protocol was designed to improve the prognosis of patients with all grades of angiocentric immunoproliferative lesions. Four patients have been entered on study. One is too early to evaluate. The other three have achieved a complete response. More patients and longer follow-up are necessary to draw any conclusions.

PHS 6043 Rev. 1-81

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI

OBJECTIVES

The objectives of this study are:

1. To determine the disease-free survival and overall survival of patients with all grades of angiocentric immunoproliferative lesions (AIL) treated with aggressive combination chemotherapy, ProMACE-CytaBOM.

2. To examine the immunologic phenotype and molecular markers of clonality in these patients.

3. To obtain tissue from these patients in an effort to establish cell lines and analyze the tissue for transforming genes.

METHODS EMPLOYED

Previously untreated patients with a tissue diagnosis of angiocentric immunoproliferative lesion (AIL), with pathology confirmation by Dr. Elaine Jaffe, Laboratory of Pathology, NCI, will undergo staging evaluation in the Biological Response Modifiers Program. Patients with any grade of AIL will be admitted to the protocol. Staging evaluation will include chest roentgenogram, CT scan of chest and abdomen, lymphangiogram, liver/spleen scan, bone scan, bilateral bone marrow aspirates and biopsies, and liver biopsy. Other tests as indicated will be performed. Patients will be treated with ProMACE-CytaBOM. ProMACE drugs (cytoxan, adriamycin, and etoposide) will be given on day one of each cycle. CytaBOM drugs (cytarabine, bleomycin, oncovin and methotrexate) will be administered on day 8 of each cycle. Prednisone will be given days 1 through 14. No treatment is given on day 15. Prophylactic Bactrim is taken throughout the entire treatment in an effort to prevent Pneumocyctis carinii pneumonia. Each cycle is 21 days long. Patients receive a minimum of 6 cycles of therapy, and 2 cycles beyond a complete response. Treatment beyond 6 cycles is necessary for patients who do not achieve a complete response by the completion of the fourth cycle. Patients who have achieved a complete response are followed at monthly intervals for the first 6 months, every other month for 6 months, every 3 months for the next year, twice yearly for the next year, and yearly thereafter.

MAJOR FINDINGS

This is a collaborative study between NIAID, BRMP and the Laboratory of Pathology, NCI.

Four patients with Grade III AIL's have been entered to this study. One patient is too early to evaluate. Each of the other three patients has achieved a complete response. None have relapsed. The duration of the complete responses are 12+, 6+ and 2+ months. Obviously, the number of patients is too small and the follow-up period is too short to be able to draw any conclusions from this study now.

SIGNIFICANCE

This trial was designed to improve the long-term survival of patients with these rare diseases. The study has evolved from an observation made within the NIH (NCI and NIAID) that patients with Grade III AIL (or angiocentric lymphoma), who were treated with aggressive combination chemotherapy had a higher response rate and improved survival compared to patients with Grades I or II AIL treated with low-dose alkylating agents and/or prednisone. Half of the patients on low-dose chronic therapy eventually developed lymphomas refractory to treatment and most eventually succumbed to their disease. Thus patients initially diagnosed with overt lymphoma enjoyed a greater long-term survival than patients with diseases (grades I and II AIL) traditionally felt to be premalignant or benign inflammatory conditions. This study is intended to determine if all patients with AIL will benefit from initial intensive combination chemotherapy.

PROPOSED COURSE

This study remains open to patient accrual. We hope to accumulate a sufficient number of patients with these rather rare diagnoses to be able to demonstrate an improved survival compared to historical controls.

PUBLICATIONS

Lipford EH Jr, Margolick JB, Longo DL, Fauci AS, Jaffe ES. Angiocentric immunoproliferative lesions: a clinicopathologic spectrum of post-thymic T-cell proliferations. Blood 1988;72:1674-81.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09342-01 CRB

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Treatment of Stage I Diffuse Aggressive Lymphomas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	P. L. Duffey	Research Specialist	OAD, NCI
	BRMP Staff		BRMP, NCI

COOPERATING UNITS (if any)

Radiation Oncology Branch, NCI (E. Glatstein), Laboratory of Pathology, NCI (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Radiation therapy in laparotomy-staged patients with Stage I diffuse aggressive lymphomas is moderately successful. This study was designed to improve upon the efficacy of radiation therapy and to eliminate the need for staging laparotomy. In this study, clinical Stage I and IE patients are treated with four cycles of ProMACE-MOPP chemotherapy (at approximately 75% of the dosages of myelosuppressive drugs originally used in ProMACE and MOPP) followed by involved field radiation to 40 Gy. There are forty-seven evaluable patients entered on study of whom forty-five (96%) achieved a complete remission. There have been no relapses. Three patients died: the two who never achieved a complete response, and one woman who had been in complete response for over four years died during her second coronary artery bypass surgery. The treatment is well tolerated and there have been no serious long-term toxicities with a median follow-up of four years. It would be difficult to improve upon these results or to decrease the toxicities.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI
BRMP Staff		BRMP, NCI

OBJECTIVES

1. To determine the complete response rate, disease-free survival and overall survival of clinically staged patients with Stage I and IE diffuse aggressive lymphoma treated with ProMACE-MOPP and involved-field radiation.

METHODS EMPLOYED

Previously untreated patients with a histologically-documented diagnosis of diffuse aggressive lymphoma, who after complete clinical staging work-up are found to be Stage 1 or IE are eligible for this study. Staging tests include chest x-ray, CT scan of chest and abdomen, liver/spleen scan, bone scan, bilateral bone marrow aspirates and biopsies, and liver biopsy. Other tests as indicated will be performed. At the completion of staging, patients will receive four cycles of modified doses of ProMACE-MOPP. ProMACE drugs are given on day 1 of each cycle: Cytoxan 500 mg/M2 iv, Adriamycin 20 mg/M2 iv, and Etoposide 90 mg/M2 iv. MOPP drugs are given on day 8 of each cycle: nitrogen mustard 5 mg/M2 iv and oncovin 1.4 mg/M2 iv. Procarbazine 75 mg/M2 p.o. is given on days 7-15 and Prednisone, 60mg/m2 p.o., is given days 1-14, days 7-15. On day 15, methotrexate 120 mg/M2 iv is administered with leucovorin rescue 25 mg/M2 po q. 6 hours for 4 doses beginning 24 hours after methotrexate. Each cycle is 28 days. Patients receive four cycles followed by 40 Gy involved-field radiation.

MAJOR FINDINGS

Fifty-one patients have been entered on this study. Two are currently receiving chemotherapy and are too early; two patients completed less than 2 cycles of chemotherapy for reasons unrelated to treatment and are also inevaluable. Forty-five of forty-seven (96%) patients achieved a complete response and none have relapsed. Three patients died: the two patients who never achieved a complete response, and one woman who had been in CR for over four years died during her second coronary artery bypass surgery. The treatment is extremely well tolerated. Forty percent of patients had no dose reductions and 41% had only one to three dose reductions. Side effects with this therapy are minimal. There were only 9 hospital admissions out of 188 courses of therapy given.

SIGNIFICANCE

With a complete response rate of 96% and no relapses after a median follow-up of over 4 years, this regimen appears to be quite effective. It would be difficult to either improve on the response rate or decrease the toxicity. Therefore,

for clinically staged patients with Stage I diffuse aggressive lymphoma treatment with full doses of ProMACE-MOPP followed by involved field radiation may be optimal therapy. Patients will be followed for analysis of late toxicities.

PROPOSED COURSE

This study will remain open, not for active recruitment of patients to the study, but only for the occasional patient who after being evaluated for the advanced stage study, is found to have early stage disease. We would like to be able to treat these patients at the NCI rather than send them back to their referring physicians.

PUBLICATIONS

Longo DL, Glatstein E, Duffey PL, Ihde DC, Hubbard SM, Fisher RI, Jaffe ES, Gilliom M, Young RC, DeVita VT Jr. Treatment of localized aggressive lymphomas with combination chemotherapy followed by involved-field radiation therapy. J Clin Oncol:1989;7:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09343-01 CRB

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Autologous Bone Marrow Transplantation in Refractory Diffuse Aggressive Lymphomas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. L. Longo	Associate Director	OAD, NCI
Others:	P. L. Duffey	Research Specialist	OAD, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	W. Wilson	Special Assistant	OD, NCI

COOPERATING UNITS (if any)

Radiation Oncology Branch, NCI (E. Glatstein); Laboratory of Pathology, NCI (E. Jaffe)

LAB/BRANCH: Clinical Research Branch

SECTION:

INSTITUTE AND LOCATION: NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with advanced stage diffuse aggressive lymphoma who do not achieve a complete response or relapse from a complete response have had a uniformly fatal outcome. This study was designed to improve upon that outcome by using high dose chemotherapy, total body irradiation, and autologous bone marrow transplantation (ABMT). All patients with advanced stage diffuse aggressive lymphomas who were treatment failures from one of several of our protocols were eligible for this study. Patients were treated with combination chemotherapy to a complete response or to a 90% partial response, bone marrow was harvested and patients then received ablative chemotherapy consisting of high-dose cytoxan followed by total body irradiation. Bone marrow treated with anti-B1 and complement was reinfused and patients were followed closely through neutropenia until marrow engraftment. A total of 23 patients have undergone transplantation with 21 achieving a complete response. Two patients were found to have residual lymphoma immediately following transplant. One of these patients was converted to a complete response with surgery and additional radiation and remains in complete response at greater than four years. There have been 7 relapses (33%). A total of 12 patients have died; seven had no evidence of lymphoma although five were treatment-related. Overall, of the 23 patients, ten patients are alive and continue in complete remission. This study demonstrates a significant improvement in survival in this group of patients; however there is a clear need to reduce treatment induced toxicities to further improve survival.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, NCI
Patricia L. Duffey	Research Specialist	OAD, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Wyndham Wilson	Special Assistant	OD, NCI

OBJECTIVES

1. To determine the efficacy of high-dose cyclophosphamide and total body irradiation followed by autologous bone marrow transplantation in patients with refractory diffuse aggressive lymphoma whose tumor burden has been reduced to a complete remission or 90% partial remission by the administration of ProMACE chemotherapy or VP16-213 and Platinum.

2. To determine the complete response rate in patients with diffuse aggressive lymphoma retreated with ProMACE chemotherapy following relapse from a complete response induced with a ProMACE-containing regimen.

3. To determine the response rate and toxicity of VP16-213 and platinum chemotherapy in patients with diffuse aggressive lymphoma who do not attain a complete response with ProMACE.

4. To determine the safety of purging the bone marrow with anti-B1 monoclonal antibody and baby rabbit complement.

METHODS EMPLOYED

All patients with diffuse aggressive lymphoma who had been previously treated on one of our aggressive lymphoma protocols, thus are uniformly treated, who relapsed from a complete remission or who never achieved a complete remission are eligible for this study. Prior to study entry, all patients are completely restaged. Those who relapsed from a CR are reinduced with ProMACE chemotherapy: cyclophosphamide 650mg/M^2 iv days 1, 8; adriamycin 25mg/M^2 iv day 1, 8; VP16-213 120mg/M^2 iv days 1,8; prednisone 60mg/M^2 iv days 1-14; methotrexate 1.5gm/M^2 iv over 12 hours on day 14 with urine alkalinization; citrovorum factor 50mg/M^2 iv beginning 24 hours after the initiation of the MTX infusion, followed by oral citrovorum every 6 hours for 4 doses or until plasma methotrexate levels have reached a safe level. Each cycle is 28 days. Patients are treated to one cycle beyond a complete response or to a stable 90% partial response. Patients who never achieved a complete response are treated with VP16-213 and platinum: VP16-213 100mg/M^2 iv daily for 5 days; platinum 40mg/M^2 daily for 5 days. Each cycle is 28 days and patients are treated to complete response or 90% PR and then one additional cycle. Patients who do not achieve an adequate response are treated with other investigational therapies.

Bone marrow is harvested from patients when they achieve a CR just prior to the last cycle of reinduction chemotherapy in order to prevent unnecessarily long delay prior to starting ablative therapy. The marrow cells are treated with anti-B1 and complement in an effort to purge the marrow of any malignant B-cells that may be present but undetected by morphologic examination. The marrow is frozen until time of reinfusion.

Following reinduction chemotherapy, patients with a complete response or 90% PR, receive ablative therapy: High-dose cyclophosphamide 60mg/kg iv on days 1 and 2 with aggressive hydration; total body irradiation 200Rad twice a day, days 3,4 and 5. Marrow is reinfused on day 6. Patients are supported through the period of pancytopenia with transfusions, antibiotics and other measures as necessary.

MAJOR FINDINGS

A total of 23 patients have undergone transplantation. Twenty-one achieved a complete response. Two were found to have residual lymphoma following transplant: one of those patients was converted to a complete response with surgery and additional radiation therapy and remains in CR lasting longer than 4 years; the other partial response was reinduced with VP16-213/platinum, achieved a CR, but died from sepsis. Of the twenty-one CR's, seven relapsed. There have been twelve deaths: five relapsed patients died with progressive lymphoma; 7 patients died with no evidence of lymphoma. Five of these deaths were treatment related; three were septic deaths that occurred during the treatment. Overall, ten patients are alive and continue in complete response.

SIGNIFICANCE

This study demonstrates that about half of the patients who come to transplant enjoy a prolonged disease-free survival. This outcome represents a significant improvement in survival for these patients who previously had a uniformly fatal prognosis.

PROPOSED COURSE

This study remains open and continues to accrue patients.

PUBLICATION

There have been no publications regarding this study.

SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1988 - September 30, 1989

I. Introduction and General Organization

The Developmental Therapeutics Program (DTP) is a comprehensive program of the Division of Cancer Treatment (DCT) charged with the discovery and preclinical development of new anticancer and anti-HIV agents for introduction into clinical trials. The DTP utilizes both intramural and extramural (grants and contracts) components to accomplish its research-oriented and investigative mission. The Program is comprised of eight extramural branches, four intramural laboratories and an applied R&D group [Program Development Research Group (PDRG)] organized directly under the Associate Director. The extramural branches include the Drug Synthesis & Chemistry Branch, Natural Products Branch, Biological Testing Branch, Information Technology Branch, Pharmaceutical Resources Branch, Pharmacology Branch, Toxicology Branch, and Grants & Contracts Operations Branch. The intramural laboratories include the Laboratory of Biological Chemistry, Laboratory of Biochemical Pharmacology, Laboratory of Medicinal Chemistry, and Laboratory of Molecular Pharmacology. The overall Program is managed from the Office of the Associate Director. The current progress of potential new clinical drug candidates through the DCT/NCI Decision Network (Drug Development) process is summarized in Table 1.

II. Program Accomplishments

A. Office of the Associate Director

1. Extramural Program Special Initiatives

Antitumor drug screens. DTP is implementing a novel primary anticancer drug screen employing a broad in vitro panel of human tumor cell lines for discovery of compounds with potential antitumor activity and possible "disease-oriented" (i.e., tumor type) specificity. In the progression from small-scale research and development to a full-scale screen for testing of >10,000 new substances per year, DTP has been using a pilot-scale screen to evaluate a number of key issues. The pilot screening panel consists of 60 human tumor cell lines representing lung, colon, melanoma, renal, ovarian, CNS and leukemia/lymphoma. All cell lines are inoculated onto 96-well tissue culture plates on day 0, in the majority of cases at 20,000 cells/well, then preincubated for 24 hours. Test agents are then added in

five 10-fold dilutions from the highest soluble concentration, and incubated for a further 48 hours. For each test, a dose response is generated for each cell line. "Standard agents" are run routinely to monitor consistency of the assay. Currently, selected for the cell growth/viability assay is a protein stain, sulforhodamine B (SRB) which binds to basic amino acids of cellular macromolecules. For this assay, cells are fixed in situ at the end of the drug incubation, washed five times and dried. SRB is added, washed five times and dried. The bound stain is solubilized and measured spectrophotometrically on automatic plate readers interfaced with microcomputers. Currently, screening is proceeding at a rate of 200 compounds per week in 60 cell lines, and will shortly expand to 400 per week.

A special screening study was used to compare the MTT assay with the SRB assay. In both assays, the cell lines were incubated for 24 hours followed by 48 hours with the test compound. 243 compounds were tested at five dose levels (with $\log_{10}$ intervals) against up to 38 human tumor cell lines, from a broad spectrum of disease types, with both MTT and SRB. Differences in cell survival percentage, between the two assays, were determined. Also, for each compound, the results of the two assays (for the ensemble of cell lines and doses) were compared using Spearman's Rho, the correlation coefficient of the ranks, and Kendall's K_C, a measure of concordance of the ranks. Compounds tested against less than 10 cell lines (5% of total) or uniformly inactive (30%) were excluded. The results of this study indicated that under the experimental conditions employed, and within the limits of the data analyses, the assays performed quite similarly; however, SRB had superior practical advantages for large-scale screening.

The ultimate configuration of the screening panel as currently planned will encompass 100 or more cell lines representing 10-12 different cancer types. Currently, a total of 60 human tumor cell lines derived from seven cancer types, which adequately meet minimal quality assurance criteria (testing for mycoplasma, MAP, human isoenzymes, karyology, in vivo tumorigenicity), which are adaptable to a standard growth medium and which have reproducible profiles of growth and drug sensitivity, have been selected for DTP screening operations during 1989. While many of these cell lines are well known and widely used, the clinical history and original tumor pathology of many such lines are incomplete or unavailable. Cell lines in the interim panels have been subjected to specialized characterizations (e.g., histopathology and immunocytochemistry) to verify tissue and tumor type. Strategies have been implemented for the development of new lines directly from surgical specimens or from nude mouse xenografts whose clinical backgrounds are more complete. Special focus is being placed upon cancer

types which currently lack adequate panel representation (e.g., breast and prostate). It is anticipated that a minimum of 70 or more new lines must be established for necessary expansions and refinements of the composition of the cell line panels.

Over 30 of the in vitro human tumor panel lines of the NCI "disease-oriented" drug discovery program have been adapted and characterized for growth as xenografts in athymic nude mice for secondary drug evaluation models. They exhibit tumor "take" rates of 80-100% from 40-mg fragment implants. Colon and melanoma lines have been the most readily established in vivo, and CNS tumors have presented the greatest difficulty. Successful sc models among CNS tumor lines include SNB-19, SNB-7, TE-671, SF-295, and U251l1. The volume doubling time (Td) between tumor sizes of 0.1 and 1.0 g ranged from about two days for SF-295 to about seven days for SNB-19. The numbers of other sc xenograft models developed to date and their Td values are: six renal cell carcinomas, 6.6 to 11.5 day; six colon adenocarcinomas, 2 to 5 days; seven melanomas, <2 to >10 days; four ovarian carcinomas, 3 to 6 days; four nonsmall cell lung carcinomas, 6 to 8 days; and three small cell lung carcinomas, 6 to >11 days. Selected lines have already been used for in vivo evaluation of candidate drugs; e.g., H82 small cell lung carcinoma xenografts have been used to evaluate the antitumor activity of penclomedine. The availability of these xenograft lines is an important complement of the present NCI drug evaluation and development strategy.

Anti-HIV drug screen. Standardization and further refinement of the primary (large-scale) in vitro anti-HIV drug screen has continued, and screening operations have progressively increased. As of May 1989, approximately 30,000 screening tests had been performed, and the testing rate was approximately 400 samples per week. During 1989 this rate will increase to approximately 1,000 samples per week. Over 8,000 synthetic compounds and 10,000 natural product extracts had been tested as of May 1989. From these approximately 130 pure compounds and 150 extracts had been found to be active. Among the active pure compounds were represented new members of six already-known active anti-HIV chemical classes. However, more importantly, new active lead compounds and related derivatives have been discovered in ten chemical classes not heretofore associated with anti-HIV activity. Among these are two new active chemical classes (sulfolipids; phorbol esters) identified in the DTP natural products drug discovery program. Development and implementation of "stage II" assays for more detailed and/or mechanism-oriented biological evaluations are continuing, and all of the new leads identified by the primary screen are being profiled accordingly.

Natural products drug discovery program. A program-wide effort for new drug discovery from natural products continues to undergo development and implementation. The focal point for this complex, interdisciplinary effort is at the Frederick Cancer Research Facility (FCRF), and involves the key participation of DTP extramural and intramural components, as well as on-site contractors at the FCRF and off-site acquisitions contractors and subcontractors operating worldwide. Scientific coordination and direction of this project is accomplished through the efforts of the newly formed DTP Natural Products Program Committee chaired by the Associate Director for DTP. More detailed descriptions of specific activities encompassed in the DTP natural products drug discovery program can be found under the separate reports for: Natural Products Branch (plant, marine and microbial product acquisitions; extraction laboratory; repository); Drug Synthesis & Chemistry Branch (synthetic chemistry support; pure compound acquisitions); Biological Testing Branch (screening operations); Information Technology Branch (data management); and the PDRG (below).

2. Intramural program initiatives (Program Development Research Group; PDRG)

The mission of the Program Development Research Group is to carry out a program of basic and applied research supportive of and complementary to the new drug research and development programs of the National Cancer Institute. To this end, the PDRG investigators report progress on a number of interrelated areas.

Assay development for the primary antitumor screen. The SRB cell protein assay was extended to accommodate suspension cultures. This was accomplished by cross-linking cells to their plastic substratum by adding an aliquot of 80% TCA to the culture medium.

Optimized protocols were developed for the Orange G, Chromotrope 2R, and Bromophenol Blue cell protein stains and for the Thionin, Azure A, Toluidine Blue O, Safranin O, and Phenosafranin cell biomass stains. All of these dyes were found to provide an acceptable basis for quantifying drug effects on cell growth and cytotoxicity in 96-well microtiter plates.

Development was initiated of a simpler and faster assay using fluorescent dyes. An excitation/emission wavelength combination was identified for propidium iodide which was insensitive to protein concentration. This allowed cellular polynucleic acids to be measured without interference from the serum protein of the growth medium. The assay is a two step process. A propidium iodide-ionic detergent mixture is added directly to the growth medium of a culture, and

fluorescence intensity is then read when cellular digestion has been completed. There are no other handling steps in the procedure. In bulk cultures and 24-well plates, the method is 2-3 times more sensitive then the SRB assay, has fewer steps, is completed more rapidly, permits a greater number of samples to be processed per unit time, and allows data entry and pattern recognition analysis to be competed the same day that samples are collected. The assay will be extended to the 96-well microtiter format when a fluorescent plate reader becomes available.

Validation and optimization studies were conducted for MTT tetrazolium assay. Reaction kinetics were determined and a Lineweaver-Burke analysis performed. The assay did not fulfill the minimum requirements of a valid enzyme assay. An excess substrate concentration could not be achieved within the solubility limits of MTT. Initial velocities produced unacceptably low optical density signals with some cell lines, and could not be read within the time range of linearity in a high volume screening mode. Apparent IC50's determined with popular protocols from the literature differed by up to 50-fold from the IC50 values determined from true initial velocity at maximum achievable MTT concentration. In addition, the reaction was not linear with cell number.

Analysis of disease-specific selective antitumor drug activity. A dedicated computer workstation has been constructed to identify drugs which act selectively on specific subpopulations of the in vitro human tumor lines which comprise the NCI's In Vitro Anticancer Drug Screen. The system consists of an HP Vectra 386 computer with a 300 Mb hard disc operating under DOS. It uses a dBase III engine to manage the Screen's data base. Drug selectivity is currently assessed by four separate criteria: maximum response, growth inhibition, cell killing, and a nonparametric rank analysis. The analytical software has recently been translated into C, can now produce a full analytical summary of an entire screening run (40 drugs x 58 cell lines x 5 test concentrations) in about 7 minutes and a complete printout of all calculations, tables, and graphs in about 40 minutes. Screening data for the 4th quarter of 1988 have been analyzed, and a short list of novel lead compounds has been identified.

Stage II testing of AIDS antiviral compounds. One of the major goals of the PDRG is to develop second-stage assay systems to expedite the characterization of compounds which have shown activity in large-scale primary evaluation. A biological safety level 3 (BL-3) facility has now been established and made functional in the PDRG. In this laboratory, much progress has been made in the development of rapid quantitative microassays for the second-stage study of anti-HIV compounds. These assays involve three general

categories: (1) confirmation of antiviral activity, (2) mechanisms of action, and (3) cellular and viral range of activity.

In addition to developing quantitative tetrazolium and fluorometric assays for the overall cytopathic effects of HIV, microculture assays measuring the effects of compounds on virus/target cell interactions, reverse transcriptase, and infectious viral release were also developed. Quantitative measurements of viral antigen expression at the single cell level were accomplished using immunocytofluorometric methods. Antiviral compounds with known mechanisms of action and comparative methodologies were used for assay validation. Recent progress in using these newly developed methodologies has allowed for the comparative testing and activity ranking of analogs of anti-HIV nucleosides and suramins, as well as second-stage testing of several newly discovered natural product antiviral agents. In addition, collaborative studies within and outside of the PDRG have resulted in the tentative establishment of the mechanisms of viral protection of the natural product prostratin. The proposed mechanism, which involves the down-regulation of the $CO4^+$ receptor on the susceptible T-lymphoblastoma cells, has been made possible in part by the development of several new assays in the BL-3 facility of the PDRG.

Work is currently in progress to expand, and fully characterize, a range of assays for the rapid determinations of the mechanisms of action of antiviral compounds. Understanding these mechanisms will aid in the selection and prioritization of novel anti-HIV agents for clinical trials.

Problems encountered with tetrazolium-based assays for estimation of cellular viability. The two tetrazolium reagents, MTT and XTT, have been extensively evaluated for use in high-flux *in vitro* antitumor screening against human tumor cell line panels. Several problems were identified which affect the reduction of XTT or MTT to their respective formazans. With XTT/PMS, variations in the pH of the RPMI 1640 growth medium, typically caused by temporary removal of culture plates from a 5% CO_2 environment, resulted in formation of crystalline material causing erratic optical density measurements. Crystal formation occurred in the pH range of 7-9 and could be attributed to reaction of PMS with the cellular thiol glutathione. For either MTT or XTT, tetrazolium reduction is dependent upon the cellular generation of NADH and NADPH. Since these reduced pyridine nucleotides are produced, in part, via glucose metabolism, an investigation into the role of medium glucose concentration on MTT reduction was undertaken in cell lines representing seven tumor histologies (CNS, colon, lung, melanoma, renal, ovarian, and leukemia). The results of these studies indicate that a progressive reduction in MTT

specific activity (MTT formazan formed/μg cell protein) was paralleled by a progressively decreasing glucose concentration. This was particularly evident for several of the cell lines (SN12K1, renal; HOP-62, lung; HT-29, colon) where a 3 - 5-fold reduction in specific activity occurred over a 24 - 48 hour period during the course of the assay and was observed to be independent of cellular viability.

Development and evaluation of a CO_2 - independent culture medium for use in in vitro anticancer drug screening. Removal of cells from a 5% CO_2 incubator required to buffer bicarbonate-based growth medium causes alkalinization of the medium and results in exposure of cells to non-physiological pH. This affects cell growth, drug activity and produces extracellular crystals which compromise the XTT tetrazolium assay. In an attempt to eliminate this increase in pH, a eukaryatic growth medium was successfully developed for use in atmospheric CO_2 in a high-flux anticancer drug screen employing human cell lines representing 7 tumor histologies (CNS, colon, leukemia, lung, melanoma, ovarian and renal). The medium derives its buffering capacity primarily from β - glycerophosphate, exhibits a stable physiological pH of 7.3 to 7.4 and was optimized to facilitate growth in atmospheric CO_2 by inclusion of biotin, L-asparagine, pyruvate and oxaloacetate. The new medium (PDRG-I) was shown to be suitable for use in both metabolically-based tetrazolium assays, as well as the sulforhodamine B protein assay for estimation of cellular viability. Similar IC values for BCNU, adriamycin and tamoxifen were obtained in cell lines maintained in atmospheric CO_2 and RPMI 1640 medium under a 5% CO_2 environment. It is anticipated that the new medium will be incorporated into the antitumor drug screening effort in the near future since nearly 60 cell lines have been adapted for growth in PDRG-1.

Assessments of various formats of soft agar culture and methods of analysis for Stage II/Stage III in vitro drug evaluations. Specific objectives of these investigations were (A) to compare colorimetric and morphometric methods of analyzing growth and drug sensitivity; (B) to compare drug sensitivity profiles of single cells and multicellular growth units (MGU); and (C) to assess the suitability of the soft agar culture format for a variety of human tumor cell lines.

Results of A investigations indicate that 1) image analysis and formazan colorimetry exhibit excellent linear regression for growth as well as drug sensitivity measurements, that 2) tetrazolium/formazan colorimetry provides an expedient means to measure overall growth and drug sensitivity in soft agar culture, and that 3) morphometric and colorimetric endpoints remain stable for a period of two weeks following culture termination, provided that dishes are appropriately rinsed and refrigerated.

Subsequent B investigations indicate that 1) the cultivation of MGU in soft agar culture are well-suited to image analysis of drug sensitivity over a multiple log range and that 2) cultures containing MGU at the time of drug application result in more discriminating indices of drug effect than cultures containing single cells.

Finally, C investigations demonstrate that MGU in soft agar culture can be generated for many human tumor cell lines: suitable growth rates and cloning efficiencies were observed for 13/21 lung, 12/13 colon, 10/11 CNS, 6/10 kidney, 13/13 melanoma, 4/7 ovary, and 5/5 leukemia cell lines. Soft agar cultures of cell lines are well-suited not only for detection and isolation of clones which survive drug exposure, but also are amenable to a variety of histologic procedures.

Tumor cell line acquisition/development. As an extension of the initial scheme for stepwise cultivation of cryopreserved cell stocks, quality assurance testing, and growth/drug sensitivity testing of human tumor cell lines (Cancer Res. 48: 589-601, 1989), preparative culture protocols are under development for specialized *in vitro/in vivo* characterizations.

These protocols include: (1) the cultivation of cells as adherent monolayers for immunocytochemistry and ultrastructural analysis, (2) the preparation of cultured cells for prostanoid analysis, and (3) the cultivation of three-dimensional colonies in soft-agar matrix for purposes of "clinical" histopathology examination and comparisons with that of fresh tumor specimens.

In addition to the characterization of cell lines already acquired by the Developmental Therapeutics Program to date, the PDRG cell culture area has also acquired a total of 28 new cell lines from a variety of sources and has assembled relevant patient and cell line histories. In addition PDRG cell culture staff have initiated cryopreserved stock preparations, culture photography, and initial quality assurance testing on each of these "priority" cell lines.

Morphological and immunochemical characterization of tumor cell lines. Cell lines derived from human carcinomas are widely used for the study of human tumor biology, and experimental therapeutics. The validity of these cell lines as disease-oriented models is dependant, in part, on the degree to which they retain the characteristics of their respective parent cancers. The present study was conducted to delineate the morphologic and immunocytochemical characteristics of candidate cell lines for the *in vitro* anticancer drug screen. Over 100 commonly used human carcinoma cell lines derived from cancers of the central nervous system, colon, lung, ovary, kidney, and from

melanomas were analyzed. Cell lines were grown in monolayer culture, and when possible, in soft agar culture and in nude mice as xenografts. Histologic, histochemical and electron microscopic examinations were performed. In addition, immunocytochemical assays for a number of markers, including various intermediate filaments, carcinoembryonic antigen, as well as panels of tissue specific antigens, were performed on material from monolayer cultures. The characterizations performed provided substantial and important information on the tissues of origin, tumor type, degree of differentiation and tumor biology of the cell lines cultivated under the in vitro and in vivo propagation techniques used in this laboratory. Ten to 20% of the cell lines were diagnosed as undifferentiated carcinomas and could not be further classified. The majority of the other cell lines were very poorly differentiated, but retained sufficient morphologic or antigenic characteristics to allow classification consistent with that of the parent cancer, although representing the more poorly differentiated elements of such a tumor. Fewer than 20% of the cell lines were found to retain the spectrum of characteristics generally associated with the parent cancer type. The tumor types of the cell lines selected for screening panels, as indicated by the characterizations, was consistent with the reported tumor histopathology. Information on degree of differentiation and other characteristics of the parent tumors for these panel lines was usually not available, and therefore, this new data represented valuable additional information on the nature of the cell lines. These findings stress the importance of detailed characterization of cell lines to determine their suitability for particular experiments, and underscore the need for development of new cell lines which represent valid models for the in vitro study of human cancer.

Biochemical characterization of lung cancer cell lines; prostanoid biosynthesis and metabolism. The prostaglandins and related lipids have been implicated as mediators of a variety of pathophysiological aspects of malignant disease (tumor metastasis, host immunoregulation, regulation of cell proliferation, etc.). A systematic evaluation of the capabilities of human tumor cells to selectively synthesize prostanoids and other lipid mediators has been undertaken for initial determination of the biochemical capabilities of human tumor cells.

The profiles of fatty acid cyclooxygenase products synthesized from endogenous and exogenous arachidonic acid have been determined in 28 established cell lines derived from human lung tumors, in 10 established cell lines originating from human colon tumors, two prostate tumors, five ovarian tumors, 10 renal cell carcinomas and four tumors from the central nervous system. Employing the sum of prostanoid biosynthesis and an index of prostaglandin H

synthase activity, our findings suggest that prostaglandin and thromboxane biosynthesis may be a unique feature of certain subclasses of non-small cell carcinomas of the lung. The level of prostanoid production and incidence of prostaglandin H synthase activity >2 picomoles/10^6 cells were consistently higher in established cell lines derived from human non-small cell carcinomas of the lung than in cell lines originating from small cell carcinomas of the lung, ovarian adenocarcinomas, colorectal carcinomas, adenocarcinomas of the prostate, renal cell carcinomas and central nervous system tumors. These findings represent the initial biochemical characterization of these cell lines derived from human solid tumors.

Lung cancer biochemistry, metabolism and experimental therapeutics. The current lung cancer research project attempts to interface clinical aspects of human lung cancer with basic science and applied science technologies in an effort to provide comprehensive characterization of lung tumors based on their morphological, ultrastructural, biochemical, molecular genetic and metabolic capabilities, as well as to establish new in vivo techniques for propagating and investigating the biology of human lung cancer. Current studies have shown: (1) significant differences in endogenous prostanoid biosynthesis, 4-ipomeanol (IPO) metabolism, cytochrome P450 (P450) and glutathione-S-transferase enzyme activities between matched normal lung and lung tumor tissue from individual lung cancer patients as well as among different established human lung cancer cell lines; (2) altered regulation of P450IA1, P450IVB1, and P450IPO gene expression in fresh human lung cancers as well as in established lung cancer cell lines; (3) a wide range of inter-individual variation in all the above biochemical and molecular genetic pathways in fresh normal lung and lung cancer tissue, as well as established lung cancer cell lines; and (4) the ability to propagate human lung tumors in the lungs of nude mice by novel intrabronchial and intrathoracic implantation methods which allow for improved in vivo propagation, biochemical, molecular genetic, as well as drug screening studies. An ultimate goal of this project is to provide a paradigm for individualized lung cancer characterization and pharmacoselective drug treatment based on the biochemical and molecular genetic profiles of individual lung tumors and corresponding normal lung tissue.

Isolation and identification of new active anti-HIV and antitumor agents from natural products. The natural products chemistry effort this year has focused on the isolation and identification of naturally occurring compounds which possess anti-HIV properties. This work involves fractionating crude extracts which were found to inhibit HIV infection in the in vitro XTT screen. All sub-fractions of an active crude extract are also assayed in

the screen and in this way the biological activity is monitored throughout the isolation process. Using a variety of chromatographic separation techniques, those fractions which retain anti-HIV activity are further fractionated. Ultimately, it is possible to obtain in pure from the individual compounds which are active against the HIV virus. The exact molecular structures of the pure active compounds are assigned primarily by analysis of their spectroscopic data. The two most important tools in structural elucidation are nuclear magnetic resonance (NMR) and mass spectrometers. These instruments provide the bulk of the spectroscopic information generally used in structural studies.

During the past year we have isolated and identified two new classes of natural product compounds, sulfolipids and phorbol esters, which exhibit striking anti-HIV properties. In both cases, the NCI/DCT Decision Network Committee has decided that these compounds are of sufficient interest to warrant Stage A preclinical development. In addition, we are currently pursuing several other projects which appear to hold exceptional promise for providing additional new classes of HIV-inhibitory compounds. During the coming year, increasing attention will be devoted to the identification and pursuit of new active natural product leads which are anticipated, as the new in vitro anticancer screen begins full operation.

B. Extramural Branches

1. Drug Synthesis and Chemistry Branch

As a key component of the DTP drug discovery effort, the responsibility of the Drug Synthesis and Chemistry Branch (DS&CB) is the acquisition of novel compounds for antitumor and anti-HIV evaluations. This is achieved through a variety of program activities. Over the past year, nearly 6,000 new compounds, both natural and synthetic in origin, were acquired for screening through ongoing scientific liaison with a network of laboratories which has been developed and maintained on a worldwide basis. A base of chemical and biological data has been accumulated on some 450,000 compounds. Most of these are stored in a DS&CB repository, which manages the receipt and shipment to screening laboratories. The DS&CB plays a key role in operation of the Acquisition Input Committee, which manages the flow of compounds through the NCI screens. About 200 pure compounds monthly are being submitted to the AIDS screen. A tracking system of 12 check points was recently devised in cooperation with the Information Technology Branch, and results on 90% of the compounds tested vs AIDS are now returned to suppliers within 4 months of submission.

Identification of structure-activity relationships by computer-assisted analysis of test results is well-established within the DS&CB.

Activity leads are optimized through the synthesis of prodrugs and congeners. Other projects are specifically for resynthesis (of compounds unavailable from the literature, and for scale-up of lead compounds) and for radiolabelled synthesis. Current efforts include the synthesis of active ATA polymers vs AIDS, nonclassical isosteres as bombesin antagonists vs cancer, and protein kinase inhibitors related to erbstatin vs cancer. Carbovir, described a year ago as a new lead vs AIDS, has now been licensed to a drug company and is under commercial development. The first total synthesis of the novel anti-AIDS product from cyanobacteria was recently completed. A novel and highly promising lead against AIDS was recently identified in a series of 450 synthetic compounds submitted by a nonpharmaceutical chemical company; this compound is being pursued actively. Another program commitment is to collaborate with DTP intramural scientists by synthesis of compounds for special projects; current examples are a series of anticancer N-mustards and CoQ analogs.

2. Natural Products Branch

The major responsibilities of the Natural Products Branch (NPB) are: 1) acquisition of crude biological materials of plant, marine, and microbial origin for the DTP anticancer and AIDS antiviral screening programs; and 2) coordination of contractual activities to support the discovery and development of new active agents from natural products.

The development of new anticancer and antiviral screens in DTP has regenerated a high level of interest in identifying new natural product leads. A major program effort in the discovery of natural products leads from a wide variety of sources, including terrestrial plants, marine invertebrates, algae and marine microorganisms, is in progress. The NPB has devoted a good deal of effort towards development of the necessary procedures, operations, and data support for the repository, extraction, drug preparation, and screening laboratories relevant to natural products.

Plant collections are progressing well in the tropical rain forest regions of Africa, Central and South America, and South East Asia, with over 12,000 samples having been collected in the past 2 1/2 years. Over 2,000 marine organisms have been collected in the Indo-Pacific region. Plant and marine organism samples are extracted at the Frederick Cancer Research Facility (FCRF), and over 7,000 extracts are currently in storage at -20°C. In the microbial area, a program for the cultivation and

extraction of marine protozoa was started recently, and new projects for the cultivation and extraction of marine anaerobic bacteria and unusual fungi will soon be initiated. A fungal cultivation contract was completed in December, 1988, and over 1,000 fungi were grown and multiple extracts provided. The program for the cultivation of cyanobacteria (blue-green algae) is making excellent progress, and a novel class of products exhibiting in vitro AIDS antiviral activity has recently been isolated by the DTP intramural isolations chemistry group. To date, over 5,000 extracts have been tested in the AIDS antiviral screen, and a number of extracts of plant, marine and microbial origin have been found to have significant in vitro activity, and are being further studied. Testing in the new anticancer screen is due to begin in the near future.

3. Biological Testing Branch

The Biological Testing Branch (BTB) has participated in the development and implementation, organization and management of a disease-oriented in vitro screening program for a large number of candidate cancer chemotherapy compounds, and for following up in vivo testing of selected agents. The BTB has also participated in the development and implementation of an anti-HIV drug screen. Further, the BTB manages a large resource for the production, quality control, and distribution of genetically and biologically defined rodents. These disease-free experimental animals are distributed to investigators at NIH, FCRF, and other investigators on a cost reimbursement basis. The BTB maintains a large repository of experimental animal and human tumor lines for usage by DCT and other NCI programs. Tumors are also distributed to qualified cancer research investigators on a cost reimbursement basis.

Quality standards for animal production have been maintained. Adjustements have been made in animal production to reflect changes in DTP objectives including more reliance on athymic mice and less overall volume of usage. The payback system has continued to work well in making the animal production system cost effective.

The tumor bank has expanded its capacity to accommodate a number of the cell lines utilized in the disease-oriented screening program. Steps have been taken to enhance the acquisition of cell lines from both the cancer and AIDS testing programs and to make these lines available for distribution.

4. Information Technology Branch

The Information Technology Branch (ITB) supports the data processing, management, and analysis needs for DTP's drug discovery and development programs in cancer and AIDS. ITB

maintains large and detailed databases for the DTP. These record information on all chemical samples tested and virtually all biological test results obtained by the NCI anticancer screen since 1956. They maintain similar databases for the AIDS anti-viral screen initiated in mid-1987.

This year much of the automated information resource was significantly modified. The main reason for change was the anticipated move of the chemistry system from the DCRT owned and operated DEC10 and IBM systems to a dedicated VAX 8820. There was also a need to enhance the integration of the screening laboratory microcomputer systems with the in vitro drug screening database. The chemistry system required extensive modification and ITB achieved this through a Phase II SBIR contract which concluded this year.

A cooperative multi-contractor effort achieved a revised in vitro drug screening database design. The group converted multiple flat files to a series of tables for use in a relational database management system. They continued to improve important microcomputer programs. A DTP staff programmer wrote a new, highly successful version of the AVAIL inventory and logistics program using dBase.

A contractor wrote a significantly improved version of the scheduling program ASSIGN and hosted it on a MicroVAX. They also devised a new free-standing version of the microcomputer-based laboratory capture and calculation program to help with the evaluation of natural product fractionation samples against HIV. There are continuing efforts to develop a similar system for the anticancer screening system.

ITB management wrote several programs of special interest this year. One of these tracks the progress of events from acquisition of samples through the screening process to generation and distribution of test results. The availability of this information together with the cooperative efforts of several DTP branches acted to minimize undue delays in distribution of screening results for the AIDS program. A similar program is under development for the anticancer effort.

ITB staff have developed techniques to express and display data from the anticancer screen as "fingerprint" patterns. These patterns can be shown to be similar for compounds related by structure or biochemical mechanism of action. ITB further refined the techniques and developed a derivative application which provides a powerful tool to monitor the reproducibility of the patterns produced. A contractor is revising these programs to run on the VAX.

The VAX 8820 was installed at the FCRF in April, 1989. During the second half of 1989, the transition from DCRT support for chemistry and in vitro screening support will be completed. While significant progress has already been made in implementation of the VAX 8820 and its integration via Ethernet with the laboratories at the NCI-FCRF, a great deal of effort will be required to ensure smooth functioning of the chemistry and biology applications and to integrate off-campus contractor and NCI staff activities.

5. Pharmaceutical Resources Branch

The Pharmaceutical Resources Branch (PRB) provides large-scale comprehensive pharmaceutical support to the Division of Cancer Treatment. High quality drugs and formulated products for investigational use are supplied for preclinical and clinical investigations on new agents for the treatment of cancer and HIV infections. The Branch consists of four functional areas through which its major activities are performed.

The chemical resources component provides for the acquisition/synthesis of new compounds. During the past year, 22 chemical products were produced in kilogram quantities through the prep-lab contract program. Several of the materials for formulation included azacytidine, BSO, SR2508, pyrazoloacridine, dihydroazacytidine, etc. Several anti HIV agents were also produced in multikilogram quantities including dideoxyinosine, dideoxycytidine, pentamidine isethionate and 2',3'dideoxyguanosine. The chemists in the chemical resource component were also involved in the isolation and purification of natural products, including bryostatin and taxol.

The analytical component is charged with all quality control assessments of bulk chemical substances, and finished pharmaceutical dosage forms. These responsibilities involve characterization and purity analyses of new chemical and natural product entities, independent analysis of finished dosage forms and shelf-life assessment of bulk substances and finished investigational dosage forms.

The pharmaceutical research and development (R&D) component converts bulk chemicals into viable pharmaceutical products for investigational clinical use. This group emphasizes new approaches to drug formulation, since chemical and physical characterizations of the discovered compounds often require significant research prior to production of a satisfactory clinical product. Dosage form development was completed on four new agents for initial IND submission: BSO, CSQ, hepsulfam and hydrazine sulfate. The R&D component continues to investigate new drug delivery systems such as liposomes, microdispersions, cyclodextrins and emulsion systems.

The pharmaceutical acquisition and production component is the largest component, including contract manufacture of dosage forms, acquisition of dosage forms, storage and distribution of dosage forms, inventories, purchasing, etc. Close to one million units of investigational products were received and distributed to investigators worldwide during this last reporting period.

6. Pharmacology Branch

The Pharmacology Branch (PB) is involved with two basic aspects in the preclinical drug developmental program: detailed therapeutic studies and pharmacokinetic studies on candidate agents for cancer and AIDS treatment. Via the contract mechanism, special studies are technically managed to explore the in vivo therapeutic efficacy of new drugs in murine models with various routes of administration and treatment schedules. For cancer drugs, advanced stage tumors, metastatic disease and drug-resistant tumors are of prime interest. For AIDS drugs, murine retroviruses (Rauscher and LP-BM5) are the current prime models but the SCID and transgenic mouse are being considered. Separate contracts are used to obtain methods of analysis for each agent of interest to our program and to conduct detailed pharmacokinetic studies in order to better understand their in vivo behavior.

Numerous cancer drug candidates were tested in detailed therapeutic studies: CPE-C (NSC 375575), Carmethizole (NSC 602668), pyrazoloacridine (NSC 366140), penclomedine (NSC 338720), 8-Cl-cAMP (NSC 614491), rapamycin (NSC 606698D) and three morpholino-anthracyclines (NSC 354646, NSC 357704 and NSC 619003D). The latter three anthracycline analogues were of particular interest because of indications in vitro for effectiveness against multidrug-resistant (Mdr) tumors. In vivo tests against the Mdr P388/Adr and the 16/C mammary adenocarcinoma have not yet demonstrated this advantage over adriamycin, although their potency is much greater than that of adriamycin.

In detailed therapeutic studies for AIDS, two activities were ongoing. A contract was put into operation to identify agents with activity against the dihydrofolate reductase from Pneumocystis carinii which was later smoothly transferred to the NIAID. Initial agents identified by the anti-HIV FCRF screen are undergoing evaluation in murine retrovirus models both in vitro and in vivo.

During this year three new AIDS pharmacology contracts and three renewed cancer pharmacology contracts were put into operation. Five novel compounds with demonstrated in vitro anti-HIV activity are currently being studied. The phosphorothioate oligonucleotides Poly-C 28-mer (NSC 613671), antisense to HIV rev gene 28-mer (NSC 613672) and a

27-mer analog (NSC 624958D) are undergoing method development as is a Uniroyal synthetic (NSC 615985D). Identification of the metabolites of the HIV-active compound carbovir (NSC 614846) continues. Five cancer agents have undergone complete method development and detailed pharmacokinetic studies, penclomedine, batracylin, histidinol, 8-Cl-cAMP, and CPE-C, while another three, cyanomorpholino, O6-methylguanine and the prodrug of rapamycin (NSC 606698) are at various stages of method development and pharmacokinetic analysis.

7. Toxicology Branch

The Toxicology Branch (TB) is charged with the responsibility for preclinical safety testing of new antineoplastic and anti-HIV drugs. This responsibility is carried out by studies in two stages, IIA and IIB, corresponding to the preclinical steps in the DCT Decision Network process. In stage IIA, the maximally tolerated dose (MTD) is determined in rodents and dogs on several schedules of administration. This year nine new antineoplastic drugs or analogs and two anti-HIV agents were in this phase of preclinical toxicology. Two drugs, CPE-C and pyrazoloacridine, moved on to stage IIB. One drug, carmethizole, was dropped due to cardiotoxicity. In stage IIB, full-scale INDA-directed toxicology studies are performed using the MTD from the earlier stage as the pivotal dose. Scheduling is established from the preclinical efficacy and pharmacology studies, the stage IIA studies and the projected clinical regimen. Two drugs completed this stage in the past year, batracylin and LBSO/LPAM.

The Branch continued two projects over the last year designed to provide more definitive information on human toxicity of investigational agents. These include studies on *in vitro* toxicity of new drugs in bone marrow committed stem cell compartments yielding a comparative hazard profile across species lines. Seven new anti-HIV drugs and four antineoplastic agents were tested comparatively on murine and human committed stem cells. In almost all cases, murine cells were more "sensitive" to the drugs than human cells confirming the safety of rodent toxicity data. A second project concerns the comparative metabolism of anti-HIV agents in lymphocytes from experimental species and humans. In the case of nucleotide analogs for the treatment of HIV infections, the agent must be phosphorylated to its triphosphate in order to be incorporated into the growing deoxyoligonucleotide chain during reverse transcriptase-directed DNA synthesis. Currently, metabolism of carbovir is being compared with that of ddI in human lymphocytes, both freshly isolated and in immortalized cell lines.

8. Grants and Contractions Operations Branch

The Grants and Contracts Operations Branch (G&COB) is responsible for administrative and managerial support for the Program-sponsored extramural activities. These activities encompass all grants, contracts and cooperative agreements.

At the end of FY 1988, 310 grants totaling $49,239,000 were administered by the Branch. During FY 1989 a total of 384 grant applications were received. About 25% are expected to be awarded by the end of the fiscal year. The subject matter of the grants ranges over the entire spectrum of biochemical, pharmacological and biological research relevant to drug discovery and development. The Program awarded 24 contracts supporting the development of antitumor and anti-HIV agents (12 for cancer, 9 for AIDS and 3 supporting both activities). In all, the Program awarded $51,938,141 worth of contracts this year as a total value over the next three to five years.

The Branch also manages and administers the National Cooperative Drug Discovery Group (NCDDG) Program through a series of cooperative agreements. This program was established in 1983 to exploit exciting new developments in biomedical research for the discovery of new and more effective anticancer treatments. Nine Groups involving 43 laboratories in 33 institutions are now supported by this mechanism at an annual cost of $4,882,000. Research projects are investigator-initiated and represent diverse areas, such as the design of inhibitors of polyamine biosynthesis, topoisomerases and growth factors. The program is being expanded by the creation of National Cooperative Natural Products Drug Discovery Groups to encourage the discovery of new agents from natural sources, such as fungi, plants and marine organisms. Awards are expected before the end of the year. In an effort to stimulate the NCDDG approach to drug discovery still further, RFAs (Requests for Applications) were released this year to solicit new applications in three areas: general mechanism of action, a disease-oriented strategy, and novel model development for the prediction of efficacy. Awards will be made to the most meritorious applications in early 1990.

B. Intramural Program

1. Laboratory of Biological Chemistry

The Laboratory of Biological Chemistry (LBC) is responsible for identifying as targets for drug design, cellular reactions that are critical to the control of tumor cell proliferation or differentiation. Recent advances in cell biology are evaluated for possible targets. Agents are

designed to interact with these targets and are evaluated for biochemical and antitumor effectiveness. An important aspect of this mission is to develop appropriate *in vivo* systems to evaluate the chemotherapeutic effectiveness of agents shown to be active in simpler *in vitro* model systems. Accordingly, the LBC is involved in identifying endogenous factors present *in vivo* that modify drug action and influence differential toxicity with the aim of manipulating these factors to enhance antitumor activity. Approximately 75% of the LBC's resources is applied to non-traditional targets for antitumor drug design and study. These non-traditional targets include early key biochemical events signaling cell proliferation or differentiation. The other 25% of LBC resources is applied to the study of either traditional targets or active compounds with traditional or unknown mechanisms of action.

Reduced requirements for stimulation by growth factors may be the fundamental characteristic of transformed (neoplastic) cells. Research in molecular biology has identified several specific biochemical changes produced by introduction or over expression of oncogenes which may reduce the levels of exogenous growth factors needed to trigger cell replication. These findings suggest that a more selective approach to chemotherapy may focus on the interaction of growth factors with cells rather than on basic metabolic reactions such as those involved in nucleic acid synthesis. We have therefore initiated projects to develop new chemotherapeutic agents to block the action of growth factors. Non-traditional targets selected for drug design and study include: second messengers inositol triphosphate and diacylglycerol; myristoylation of cellular oncogene products; protein kinase C; and selected G-proteins.

The products of two distinct oncogenes (src and ros) are associated with increased levels of phosphatidylinositol polyphosphates in the membranes of cells transformed by these oncogenes. Test systems have been developed to identify drugs that inhibit phosphatidylinositol turnover or synthesis. A series of analogs of *myo*-inositol was synthesized and evaluated. The 5-deoxy-5-fluoro-inositol analogue is incorporated into cellular phospholipid and phosphorylated to a compound similar to PIP, but not further phosphorylated to the corresponding PIP2 derivative. This system is currently being used to evaluate analogues of bombesin.

It may be possible to alter the activity of an oncogene product by interfering with its localization in the plasma membrane. Myristoylation has been shown to be critical for the membrane localization and cellular transforming activity of $p60^{src}$ and has been implicated for other transforming proteins. N-Myristoyl transferase has been further purified

from bovine brain for the purpose of preparing antibodies to be used to clone the structural gene from brain libraries. A screen of newly synthesized compounds has identified two (i.e., 1-S-CoA-2-ketopentadecane and 2-bromotetradecanoyl-S-CoA) as *in vitro* inhibitors of N-myristoyl transferase activity.

These compounds are currently being examined in the Program's AIDS screen and in cell culture in our laboratory for their effect on $p60^{src}$ N-myristoylation. N-Myristoyl- and non-myristoyl- peptides homologous to the N-terminus of $p60^{src}$ have been used to identify a high affinity (D_d = 2.7 nM) protein "acceptor" in red cell membrane vesicles. Characterization of this "acceptor" suggests that it may be responsible for the specific localization of *src* to the plasma membrane/skeleton interface and may represent a new regulatory component for the $p60^{src}$-mediated tyrosine kinase activity involved in the normal cellular regulation and transforming activity of *c-src* and *v-src*, respectively.

N-Myristoylation also appears to be a critical event in the replicative cycle of mammalian retroviruses. Both in the case of type C viruses which assemble at the plasma membrane and type D viruses which bind the inner plasma membrane following assembly in the cytoplasm, the respective N-myristoylated *gag* gene products are apparently necessary for membrane binding and subsequent viral maturation. In both cases it has been shown that blocking *gag* N-myristoylation blocks viral development. Thus, N-myristoylation represents a unique new target for anti-AIDS drug development. A newly developed method of identifying N-myristoylated proteins which were recently developed has been used to establish unequivocally $p17^{gag}$ of HIV as an N-myristoylated protein.

Investigations were continued in two interrelated projects that center around the role of protein phosphorylation in various regulatory systems. The first project deals with the role of the proto-oncogene tyrosine proteinkinase, c-*fes*, in myeloid cell differentiation. Two major studies were published this year dealing with the c-fes tyrosine kinase. The first study established that the previously purified and characterized 93 kDa tyrosine kinase of differentiated HL-60 cells was the end product of c-fes, and that this activity was associated with more highly differentiated myeloid leukemias, as well as with normal monocytes and granulocytes. The second study established that c-fes was an essential component of myeloid differentiation. The poorly differentiated myeloid leukemia cell line K562 does not express c-fes, but upon transfection with the c-fes gene acquired phenotypic characteristics of more mature cells as well as the ability to respond to a differentiation agent. The study is the first demonstration that a normal proto-oncogene is a prerequisite for myeloid differentiation. The second

project has led to an interesting characterization of the proteolytic processing of protein kinase C in Adriamycin-resistant HL-60 cells. In an initial paper, it was reported that resistant cells have an abundance of both protein kinase C and the catalytic fragment of protein kinase C, termed M-kinase, compared to wild type cells. Antibodies to protein kinase C removed the M-kinase from cell extracts and inhibited M-kinase-dependent phosphorylation in vitro. The proteolytic processing of three isoforms of protein kinase C in resistant cells has now been characterized, and it has been shown that these isoforms are processed differently. In addition, it has been discovered that the gamma isoform of protein kinase C is induced or stabilized by phorbol esters only in resistant cells. Since phorbol esters appear to enhance drug resistance, as well as activate protein kinase C, it appears that expression of specific isoforms of proteinkinase C may be important in this phenomenon.

Studies were extended on the ADP-ribosylation factor of adenylate cyclase (ARF), a recently characterized G-protein. A combination of biochemical, genetic, and immunological techniques was used to demonstrate that ARF is an essential gene found in every eukaryotic organism tested including man, yeast, slime mold, and plants. The ARF protein is structurally related to both the p21 ras oncogene family of small GTP-binding proteins, as well as the G-protein family of membrane regulatory proteins. Microinjection of ARF into Xenopus oocytes was shown to inhibit the p21 ras dependent or insulin-stimulated meiotic maturation. Further efforts are underway to determine the extent of functional cross-reactivity of ARF with the ras proteins. ARF was shown to be both physically and functionally associated with Golgi membranes, where it is highly concentrated on the cytosolic side of Golgi cisternae and appears to function as a regulator of proper protein processing. At least 7 ARF genes have been cloned and sequenced from four organisms including man, cow, yeast, and drosophila, providing a good picture of the domains of the protein that have been absolutely conserved through evolution. Most of these proteins, as well as a number of activated mutant forms of ARF, have been expressed in bacteria to allow the purification of mg amounts of protein for subsequent biochemical analyses. A detailed analysis of the nucleotide binding and hydrolysis properties of recombinant ARF has revealed a number of interesting features of the regulation of ARF activity.

It has been discovered that a nuclear protein with a molecular weight of about 55,000 is retinoylated in HL60 cells. This is a new biochemical reaction discovered in this laboratory. The chemical properties of the retinoylated covalent bond indicates that it is a thioester linkage. Thus, retinoic acid is bound most likely to cysteine. Other cell lines, including HL60 mutants, have

been examined for retinoylation. Qualitatively, the extent of retinoylation corresponds to the sensitivity of the HL60 cell line to differentiation by retinoic acid. All of the other cell lines had 3 or 4 retinoylated proteins. One of these proteins, common to all the cell lines, appears to be similar to the HL60 retinoylated protein. These results are consistent with an involvement of retinoylation in the response of cells to retinoic acid.

Flow cytometric techniques were developed to measure c-myc protein in individual HL60 cells. With fluorescence microscopy, c-myc protein was localized in the nuclei and was removed by treatment with DNase but not with RNase. In exponentially-growing cells the amount of c-myc protein/cell, normalized to cell volume, was constant throughout the cell cycle. During induction of differentiation with either DMSO or retinoic acid, decreases in c-myc protein/cell were correlated with decreases in cell growth and the appearance of differentiated cells. It was not correlated with the known decreases in c-myc mRNA.

Rat kidney NADPH-linked high-Km aldehyde reductase was shown to be a potent inducer of monocytic differentiation of HL60 cells. The enzyme activity was not required for differentiation activity and it therefore appears that the inducing activity is related to the primary structure of the protein. Aldehyde reductase was shown to bind to specific surface receptors on HL60. There are about 13,000 receptors per HL60 cell. Attempts to sequence aldehyde reductase were not successful because of a blocked N-terminus.

A new project was initiated to study density-dependent regulation of metabolic pathways. HMG-CoA reductase was studied in glial tumor cells at various cell densities. The results show that, although enzyme activity and level declines continuously from sparse to confluent densities, RNA levels only begin to change when cells are at relatively high density. Different modes of regulation may correspond to differences in cell cycle controls over C6 growth rate reported previously.

A project involving traditional targets for drug design and study is continuing to determine the relative dependency of host and tumorous tissue on de novo vs salvage pathways for the synthesis of pyrimidine and purine nucleotides in vivo. De novo pyrimidine synthesis was studied in vivo by infusing mice with L-[^{15}N]alanine and measuring by mass spectrometry the incorporation of label into the uracil nucleotide pool of liver and intestine. From these data and the measured size of the uracil nucleotide pool, the absolute amount of uracil nucleotides formed by de novo synthesis during the infusion could be calculated. The amount of uracil nucleotides formed by de novo synthesis was linear in time

for at least two hours, with the slopes giving an estimate of 29 ± 2 nmoles/gram/hour for the rate of de novo pyrimidine synthesis in liver, and 71 ± 4 nmoles/hour/gram in intestine. These results demonstrate the utility of L-[^{15}N]alanine infusions in the study of de novo pyrimidine synthesis in mouse liver and intestine.

The antitumor agent (NSC 368390, DuP 785), presently in clinical trials, has been shown to inhibit the de novo pyrimidine mitochondrial enzyme dihydroorotate dehydrogenase. In order to study the effects of this compound in intact cells, we developed a GC/MS method to quantify dihydroorotate (DHO) and orotate (ORO). L1210 cells exposed to 2 uM NSC 36890 resulted in a net positive growth, increasing the doubling time to 120 h compared to 12 h for control cells. The UTP pools in L1210 cells (0.2 x 10^5 - 5 x 10^5 cells/ml) continuously exposed to 2 uM NSC 368390 were reduced to less than 10% of control cell UTP levels. DHO in the treated cells continued to accumulate at a linear rate (8.3 +/-1.1 s.d. nmol/10^6 cells/h) over a time period of 10-70 h. Growth inhibition was prevented by simultaneous addition of uridine and NSC 368390; however, the addition of uridine after 48 h exposure of cells to NSC 368390 did not restore normal growth, and soft agar reculture of these treated cells indicated <1% of the cell population was capable of colony formation. Quantification of DHO and UTP pools in treated cells after 48 h-addition of uridine showed that DHO stopped accumulating and that UTP levels were restored. The results of these studies suggest that cells treated with NSC 368390 are metabolically active, but have been altered by a secondary effect of the compound which prevents cell division. Further studies are continuing to elucidate the mechanism of this secondary effect of NSC 368390 on cells.

Analysis of the characteristics of tryptophan antimetabolites on cell culture of L1210 murine leukemia cells revealed a unique feature of 4-fluoro-tryptophan not shared by its 5- and 6- positional isomers. The 4- isomer was still cytotoxic at concentrations equivalent to its metabolite, tryptophan, and at even higher metabolite: antimetabolite ratios where the cytotoxicity of the isomers was abolished. Cytotoxicity was accompanied by unequal growth, failure to divide and subsequent lysis. The activity of the antimetabolite in the presence of its metabolite should make it effective in vivo where natural amino acid concentration cannot be controlled. Such studies with continuous release pellets established limit levels for host (mouse) toxicity. Reports that the cytotoxicity of interferon-gamma is due to tryptophan-deficiency brought about by induction of the tryptophan degrading enzyme, indolemamine 2,3-dioxygenase, has prompted studies on combination chemotherapy with the biological response modifier and antimetabolite.

2. Laboratory of Biochemical Pharmacology

The Laboratory of Biochemical Pharmacology (LBP) is responsible for the study of the mode of action of new antitumor drugs. The LBP studies new agents which have originated within the DTP and also agents derived from extramural sources in whose preclinical development the Program is playing a major role. Over the past three years, the LBP has also participated actively in elucidation of the cellular pharmacology of compounds with anti-HIV activity, currently under development within the Program.

During the past year, the cellular pharmacology of 2',3'-dideoxy-2'-fluoro-arabinosyladenine (fddA) has been investigated in cultured Molt-4 cells. Unlike 2',3'-dideoxyadenosine (ddA), this fluorinated nucleoside is wholly resistant to decomposition by acid, and as a consequence, exhibits excellent oral bioavailability. fddA is also about 10 times more resistant to attack by adenosine deaminase than is ddA; its deamination product, fddI, is completely refractory to phosphorylytic decomposition by purine phosphorylase. The half-life of the parent drug in RPMI medium (10% calf serum) is 4 hr, which is markedly longer than that of ddA ($t_{1/2}$:15 min). The anabolism of fddA to the 5'-diphosphate and 5'-triphosphate is 22 and 5 times more energetic than the analogous processes measured with ddA. Viewed in concert, these results warrant consideration of fddA as an oral therapy for AIDS.

In the past year, in collaboration with the Clinical Oncology Branch, we have also investigated the influence of granulocyte-macrophage colony stimulating factor (GM-CSF) on the replication of HIV-1 in cells of monocyte/macrophage (M/M) lineage, and its effect on the anti-HIV activity and metabolism of several 2'3'-dideoxynucleoside congeners of thymidine in these cells in vitro. We found that replication of HTLV-III_{Ba-L} (a monocytotropic strain of HIV-1) and HTLV-III_B (a lymphocytotropic strain) is markedly enhanced in M/M, but not in lymphocytes, exposed to GM-CSF in culture. Moreover, GM-CSF reduced the dose of HIV required to obtain productive infection in M/M. Even in the face of this increased infection, GM-CSF also enhanced the net anti-HIV activity of AZT and several related congeners: 2',3'-dideoxythymidine (ddT), 2'3'-dideoxy-2'3'-didehydrothymidine (D4T) and 3'-azido-2'3'-dideoxyuridine (AZddU). Inhibition of viral replication in GM-CSF-exposed M/M was achieved with concentrations of AZT and related drugs which were 10-100 times lower than those inhibitory for HIV-1 in monocytes in the absence of GM-CSF. Other dideoxynucleosides not related to AZT showed unchanged or decreased anti-HIV activity in GM-CSF-exposed M/M. To investigate the possible biochemical basis for these effects, we evaluated the metabolism of several drugs in M/M exposed to GM-CSF. We observed in these cells markedly

increased levels of both parent and mono-, di-, and triphosphate anabolites of AZT and D4T compared to M/M not exposed to GM-CSF. By contrast, only limited increases of endogenous competing deoxynucleoside-5'-triphosphate pools were observed following GM-CSF exposure. Thus, the ratio of AZTTP/dTTP and D4TTP/dTTP is several fold higher in GM-CSF-exposed M/M, and this may account for the enhanced activity of such drugs in these cells.

Taken together, these findings suggest that GM-CSF increases HIV-1 replication in M/M, while at the same time enhancing the anti-HIV activity of AZT and related congeners in these cells. These results may have implications in exploring new therapeutic strategies in patients with severe HIV infection.

Studies continued on the biochemistry and pharmacology of tubulin, a protein critical for cell division, which is the target protein for many antineoplastic agents.

There were four major projects with antimitotic compounds in the last year: (1) the agent 2,4-dichlorobenzyl thiocyanate was found to interact with high specificity with b-tubulin, forming a covalent bond with cysteine 239, resulting in inactivation of tubulin for microtubule assembly; (2) the mechanism of action of the most promising agents derived from the South African tree Combretum caffrum, combretastatin A-4 and combretastatin A-2, were studied in detail. They were found to bind extremely rapidly to tubulin at the colchicine site and dissociate more slowly from the protein; (3) dolastatin 10, a highly cytotoxic pentapeptide derived from the sea hare Dolabella auricularia, was demonstrated to act as a potent noncompetitive inhibitor of vincristine- and vinblastine-binding to tubulin and to eliminate exchange of guanine nucleotide on tubulin. Several stereoisomers of the natural product were equally active as inhibitors of tubulin polymerization, as was a tripeptide fragment of dolastatin 10; and (4) mechanisms of binding to tubulin of colchicine analogs more active than colchicine itself were extensively studied. Enhanced activity was associated with a greater association rate constant.

The Laboratory's interest in tubulin-nucleotide interactions was carried forward in three project areas: (1) a careful evaluation of reaction conditions on the effects of nonhydrolyzable GTP analogs on tubulin polymerization was performed; (2) following reports in the literature that beryllium fluoride stabilized microtubules by acting as a phosphate analog, beryllium fluoride has been under investigation in these reaction systems; (3) the GTP analog 2',3'-dideoxyguanosine 5'-(a,b-methylenetriphosphate) was synthesized since it should act as a powerful initiator of microtubule assembly. The analog's interactions with

tubulin should provide clues to permit exploitation of the exchangeable nucleotide site as a target for microtubule disruption.

Work continued on developing methods to separate the tubulin subunits preparatively and on reconstitution of active tubulin following its denaturation. Efforts have been continued to purify a microtubule-associated protein which causes the formation of massive bundles of microtubules.

3. Laboratory of Medicinal Chemistry

The objectives of the Laboratory of Medicinal Chemistry (LMC) are the discovery and development of new anticancer, anti-AIDS and antiviral drugs. Organic, analytical and polypeptide chemistry, as well as computer-assisted molecular modeling research, are the areas emphasized to accomplish LMC goals.

Arabinosyl-5-azacytosine (ara-AC), a compound synthesized a number of years ago in this laboratory, completed Phase I clinical trials at several institutions, including both an adult and pediatric study at the NIH Clinical Center. The LMC applied the analytical methods it had developed for measuring ara-AC in body fluids to conduct pharmacokinetic studies in conjunction with both COP clinical trials. Although ara-AC is hydrolytically unstable and rapidly cleared from the plasma, steady-state levels of this drug were found to be proportional to dose and within the range (0.3-1.0 uM) reported for in vitro activity. This latter result correlates with the encouraging signs of antitumor activity observed in the adult study.

LMC interest in the antitumor and antiviral activity of cyclopentenyl nucleosides continues to be high based on continuing program preclinical development of our analogue cyclopentenyl cytosine (CPE-C) as a clinical candidate. Pharmacokinetic studies have been carried out in rats and dogs and presently continue in rhesus monkeys, using automated HPLC methodology developed in this laboratory. The long terminal phase of elimination ($t_{1/2}$ = 19-37 hr) indicates a deep compartment in which high concentrations of CPE-C or its phosphorylated metabolites might reside.

Analogues of CPE-C which might have different biochemical properties have been prepared. 3-Deaza-CPE-C, 3-deaza-CPE-U (also a 3-deazauridine analogue) and 5-aza-CPE-C have been synthesized and will be evaluated for antitumor and antiviral activity. 3-Deazaneplanocin, synthesized earlier by us, has been found to have the highest selectivity index ever observed (10,000) against rotavirus. A successful total synthesis of neplanocin F, a naturally occurring isomer of neplanocin A, was accomplished.

Analytical methodology has been developed in this laboratory to determine the bioavailability of oral HMBA in Phase I clinical studies and to compare disposition and metabolism between oral and parental dosage forms. A gas chromatographic method allows simultaneous measurement of HMBA and its major acid and basic metabolites. We have used this methodology to show that the oral drug in solution is completely bioavailable and that tablet HMBA is bioequivalent to solution HMBA in terms of plasma levels, metabolic pattern and urinary excretion.

Among the initial compounds synthesized as designed inhibitors of protein kinase C, several have been shown to be specific inhibitors of the binding of [^{3}H]phorbol-12,13-dibutyrate in the mouse cytosolic phorbol aporeceptor assay (ID_{50} 8 ug/ml).

The IMC's unique phosphoramidite synthon (containing a stabilized form of 5-azacytidine) has been used to prepare sequence specific 26-mers using a DNA synthesizer. These materials are being evaluated for their ability to inhibit DNA-methylase which should result in altered gene expression.

2'-Deoxy-2'-fluoro-2-oxopyrimidine riboside (2'-F-zebularine) was synthesized as an intermediate in the pathway to the corresponding 3'-deoxy analogue intended for anti-HIV testing. It was discovered in collaboration with the Laboratory of Biochemical Pharmacology that 2'-F-Zeb is a potent and selective inhibitor of dCMP deaminase. A compound with this mechanism of action could be useful when administered with anticancer as well as anti-AIDS drugs which are deaminated at the nucleotide level to inactive metabolites.

Oxetanocin is a naturally occurring anti-AIDS nucleoside containing an unusual four-membered oxetane ring. In an attempt to provide information on whether the 4-member ring was crucial for activity, two ring-expanded isomers were synthesized. One was active, one was not. Additional work in this series is on-going to further elucidate the structure-activity relationships in this series.

Six 2'-fluoro, acid-stable dideoxyadenosine nucleosides were synthesized in an attempt to increase the partition coefficient and enhance the CNS penetration of the anti-AIDS parent compound, 2'-F-dd-ara-A. Methyl substitution at either the 2- or 8-positions on the purine ring abolished activity. However, methyl or benzoyl substitution on the 6-amino group resulted in more lipophilic analogues which retained ca. 75% of the protective effect of ddA. Structure-activity studies are in progress.

A polypeptide laboratory has been established and an anti-AIDS project has been designed. The objective is the discovery of new anti-HIV drugs by the inhibition of the retroviral protease. The approach being used is based on the anti-sense polypeptide concept.

Molecular modeling using the hardware and software available at DCRT has provided structural insights into possible critical structural parameters found in active anticancer and anti-AIDS compounds. This technique has been extended in a prospective manner to current LMC synthetic research related to inhibitors of protein kinase C, dideoxypurine nucleosides and oxetanocin analogues.

4. Laboratory of Molecular Pharmacology

The major goal of the Laboratory of Molecular Pharmacology (LMP) is to obtain basic knowledge that could be applicable to the development of new strategies for the selective killing of human tumor cell types.

DNA topoisomerases are receiving a great deal of attention in several laboratories as potential new chemotherapeutic targets and are an ongoing focus of work in the LMP. This area of investigation grew out of studies conducted in the LMP about 10 years ago using the DNA filter elution methodology which had been developed in the Laboratory. This work had shown that several clinically active anticancer drugs, including doxorubicin, amsacrine and other DNA intercalative drugs, affect DNA in a manner that was suggestive of an effect on a DNA topoisomerase. Subsequent work in the LMP further developed and strengthened this hypothesis which was finally verified by work carried out both at Johns Hopkins University and in the LMP. In 1984, the Johns Hopkins studies showed that the enzyme topoisomerase II that they had isolated and characterized was affected in the expected manner by the drugs, a finding that greatly stimulated the field because it made possible studies in purified systems. Recent LMP studies have aimed to correlate the drug effects in purified systems with effects on intact cells or isolated cell nuclei. In addition to the DNA intercalative anticancer drugs, it has been found that the etoposides (VP-16 and VM-26) are specific inhibitors of topoisomerase II, and that camptothecin is a specific inhibitor of topoisomerase I; these drugs feature prominently in current work in the LMP.

During the current year, there was completed a structure-activity study of 35 camptothecin derivatives in which the effects on purified topoisomerase I were found to be closely correlated with previously reported activity against L1210 leukemia in mice. This provides strong evidence that effects on topoisomerase I are the cause of the antitumor activity. In addition, the structure-activity relation for

topoisomerase I inhibition indicated that the drug binds to an asymmetric site on the enzyme or enzyme-DNA complex and provided clues to the geometry of the drug-binding site. The results show where the drug molecule can be changed so as to add substituents that would increase the water solubility or otherwise alter the pharmacologic properties of the drug, without loss of activity on topoisomerase I. Since lack of solubility in water is a major limitation in the potential effectiveness of camptothecin as an anticancer drug, special attention was given to this aspect of the problem, and a derivative was identified that has increased solubility without loss of potency in topoisomerase I assays. The work has also indicated how further chemical modification could combine increased solubility with increased potency.

Several laboratories are trying to determine the mechanisms by which cells can become resistant to topoisomerase II inhibitors, and several diverse mechanisms involving alterations in topoisomerases have emerged. In the resistant cell line which is under study, a unique mechanism has emerged in which the resistance to topoisomerase II inhibitors is associated with the appearance of an altered topoisomerase I. The current evidence suggests that what is involved is an alteration in topoisomerase phosphorylation by a protein kinase. The possibility is being investigated that the resistance in these cells may be due primarily in an altered protein kinase activity.

Since nitrogen mustards such as cyclophosphamide and melphalan continue to play a major role in cancer chemotherapy, despite their nonspecific chemical reactivities, experiments are underway to determine how the selectivity of the reaction of this class of drugs might be increased so as to increase their clinical utility. These studies have been focused upon the reactions at DNA guanine-N7 positions, since these are the major reaction sites and the most likely primary sites of drug action. The dependence of reaction selectivity on the DNA sequence pattern in the neighborhood of the reaction site is being investigated. Previous studies had revealed major differences in the intensities of the reactions of nitrogen mustards dependent upon the DNA sequence around the reaction site, and had indicated that the origin of the reaction selectivity could be understood in terms of the calculated electrostatic potential at the reaction sites. It had also been found that, among seven nitrogen mustards selected on the basis of clinical usefulness or potential interest, five exhibited similar patterns of DNA sequence selectivity, while two showed unique and unusual sequence selectivities. This demonstrated that the sequence selectivity could be altered by manipulating the chemical structure of the nitrogen mustard, thereby providing new opportunities for

drug development. The two unusual drugs were uracil mustard and quinacrine mustard, and in each case it was possible to achieve at least a partial understanding of the origin of the unique selectivities in structural terms. Additionally surveyed were 35 additional nitrogen mustards from the drug inventory of the Developmental Therapeutics Program, and two new cases of unique reactivities were found and which are the subjects of current investigation. Because of the differences between DNA in free solution and DNA in nuclear chromatin, the effects of ionic strength, divalent cations, polyamines and DNA intercalators are being studied; although the overall reaction intensities are altered, the DNA sequence selectivities are remarkably resistant to these changes. This suggests that the findings with DNA in free solution may be relevant to DNA in the cell. To test this further, nucleosomes have been reconstituted on a DNA fragment and so far little change in selectivity has been detected.

The pharmacologic differences have been examined further among nitrogen mustards by determining the kinetics of DNA crosslink formation and repair in cells. Major differences were observed among the commonly used nitrogen mustards. The results suggested that it may be possible to extend classical pharmacokinetic models to intracellular compartments which would include DNA crosslinks, the likely effective pharmacologic targets, as a pharmacokinetic compartment.

The preferential repair of DNA lesions in transcriptionally active, as opposed to inactive, regions of the genome in mammalian cells has been under study. Preferential repair appears to be a crucial mechanism for the ability of cells to survive DNA damage. A survey of human cells derived from patients having various known genetic disorders of DNA repair revealed deficient preferential repair of UV lesions in Cockaynes syndrome and in Gardiner's syndrome. This is the first time that human cells have been found that are incapable of preferential repair.

New methodologies have been developed which allow these studies to be carried out on a variety of DNA lesions, and there has been verification that both nitrogen mustard adducts and cisplatin adducts are repaired preferentially in the active dihydrofolate reductase gene, as compared to inactive regions flanking this gene.

The functioning of the chromosome cycle in normal and tumor cells in proliferating and quiescent states has been under investigation. The focus continues to be the H2A histone family, which was previously identified as having several interesting characteristics. Unique among the four nucleosomal histone families, the H2A family contains species which have been conserved as separate discernable

entities during evolution. There are three such species: H2A.1, H2A.Z, and H2A.X. The cDNAs or genes for all three have now been cloned. Also cloned was the human gene for H2A.Z, the most dissimilar of these three species, and the control of the expression of this gene in proliferating and quiescent cells is being investigated. Also isolated and sequenced was the human cDNA for H2A.X. This previously unsequenced protein was thought to contain a unique carboxy-terminal region. Quite unexpectedly, the protein sequence of this carboxy-terminal region was found to be highly homologous to that of the major H2As of yeast and other lower eukaryotes. These findings indicate that the histone H2A family has unique roles in the functioning of the chromosome cycle.

The dependence of the interactions between histone and DNA on the presence or absence of DNA replication continues to be a subject of study. Methods have been developed to identify histones not bound to chromatin, and their characteristics are being investigated. Evidence has been found that these "soluble" histones are present in complexes, and the interactions of these complexes with DNA are also being investigated. These interactions between histone and DNA are important during replication of chromatin, during the functioning of chromatin in quiescent cells, and in gene transcription.

Table 1

COMPOUNDS THAT PASSED DECISION NETWORK (4/1/88 - 3/31/89)

NSC Number	Name	Compound Type*
	Decision Network IIA	
	Antineoplastic	
619679	Stannous purpurin	S
037364	O^6-methylguanine	S
615291D	Discreet	NP
619029D	Discreet	NP
	Anti-HIV	
290193	Cyclosporin A	NP
614989D	Discreet	S
615989D	Discreet	S
617692D, 619678D, and 619858D	Discreet	NP
619955D, 619956D, and 620700D	Discreet	S
621808D, 621809D, 621810D, and 621811D	Discreet	NP
620100D	Discreet	S
620103D	Discreet	S
	Decision Network IIB	
	Antineoplastic	
375575	Cyclopentyl cytosine	S
609699	Hycamptamine	SS
	Anti-HIV	
626201	Pentosan sulfate	S
	Decision Network III	
	Antineoplastic	
056410	Porfiromycin	NP
057155	Symetamine	S
123127	Liposomal doxorubicin	NP
618082	Interleukin-1	B
618085	Interleukin-4	B
620860	Xomazyme-H65	S
	Monoclonal antibodies:	
626196	Anti-TAG-72	B
626197	Anti-CEA	B

NSC Number	Name	Compound Type*
	Decision Network III	
	Antineoplastic (continued)	
	Antigen systems for monoclonal antibodies:	
626198	Melanoma	B
626199	Lymphoma	B
626200	Colon	B
	Anti-HIV	
619103D	Discreet	S
	Decision Network IV	
	Antineoplastic	
281272	Fazarabine	S
349174	Piroxantrone hydrochloride	S

* S = synthetic
NP = natural product
B = biologic
SS = semisynthetic (natural product modified synthetically)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06191-02 OAD

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Program Development Research Group

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

Biological Testing Branch, DTP, FCRF, NCI; Natural Products Branch, DTP, NCI; Program Resources, Inc., FCRF; Johns Hopkins University, Dept. of Medicine, Pulmonary Division, and Dept. of Surgical Pathology, Baltimore, Maryland

LAB/BRANCH

Office of the Associate Director

SECTION

INSTITUTE AND LOCATION

NCI, Frederick Cancer Research Facility, Frederick, Maryland

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
24	12	12

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mission of the Program Development Research Group (PDRG) is to carry out a program of basic and applied research supportive of and complementary to the new drug research and development programs of the National Cancer Institute. To this end, the PDRG investigators report progress on a number of interrelated areas. New research programs are underway in natural products chemistry, cellular pharmacology, ultrastructure, immunochemistry, tissue culture, and tumor cell biology. A sulforhodamine B (SRB) assay was developed for quantitating drug effects upon stratum attached cells, as was an MTT tetrazolium assay for single cell suspensions. A bicarbonate-free growth medium was developed. Evaluation of its ability to support the growth of cell lines in the screening panels is in progress. Nearly 200 human cell tumor lines have been evaluated as candidates for possible inclusion in the anti-AIDS and anticancer drug screens. Those which grew adequately in culture, were of early passage, and were obtained from tumors with documented pathology from patients not receiving prior chemo- or radiation therapy, are undergoing extended evaluation. The histopathology, immunochemistry, ultrastructure, growth physiology, biochemistry, and chemotype of these lines are being analyzed in detail. Novel procedures for Stage I in vitro anticancer and anti-HIV drug screening are being developed. Pattern recognition algorithms have been developed for identifying drugs which act selectively against specific subsets of tumor cell lines. Novel protocols for Stage II in vitro anticancer and anti-HIV screening are being developed, and are being optimized in a basic research mode using sets of standard clinical drugs. A number of cyanobacterial, tropical plant, and marine natural products with anti-AIDS activity were successfully fractionated. The chemical structures of several new classes of active anti-HIV agents have been elucidated, and several others are in the advanced stages of structure identification.

PHS 6040 (Rev. 1/84) GPO 914-918

Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Parameters which Affect Formazan Production. Two hydrogen acceptor reagents, 3-(4,5-dimethylthiazol-2-yl)-2,4 diphenyltetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H tetrazolium hydroxide (XTT), have been extensively evaluated for feasibility of use in high-flux in vitro antitumor screening against a broad panel of human tumor cell lines. Two potentially critical problems were encountered which prompted the present investigations. For either MTT or XTT, tetrazolium reduction is dependent upon the cellular generation of NADH and NADPH. This prompted concern about the influence of glucose concentration in the cell culture medium upon the formation of the colored tetrazolium formazan which is measured colorimetrically as an estimate of cellular growth/viability. Studies with MTT indicated that a progressive reduction in MTT specific activity (MTT formazan formed/μg cell progein) observed during the course of a typical 7-day assay was paralleled by a progressively decreasing glucose concentration. For XTT, there was a further problem due to requirement for the addition of an electron transfer reagent, phenazine methyl sulfate (PMS), to promote adequate cellular reduction of the tetrazolium. With XTT/PMS, variations in pH of the RPMI 1640 growth medium, typically caused by temporary removal of culture plates from a 5% CO_2 incubator environment, resulted in occasional formation of crystalline material causing erratic optical density measurements. Crystal formation occurred in the pH range of 7-9 and could be attributed to reaction of PMS with glutathione (pK=8.6).

Development and Evaluation of a CO_2-Independent Culture Medium for Use in a High-flux In Vitro Anticancer Drug Screen Employing a Broad Panel of Human Tumor Cell lines. A tetrazolium-based assay employing 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino)carbonyl]-2H tetrazolium hydroxide (XTT) has been evaluated for feasibility of use in estimating cellular growth/viability in a high-flux in vitro antitumor screen against a broad panel of human tumor cell lines. During the course of increasing screen throughput, formation of extracellular crystals was encountered in RPMI 1640 growth medium when culture plates were removed from a 5% CO_2 environment. In an attempt to eliminate the pH instability problem, an alternative medium was successfully developed for growth of representative cell lines from seven tumor panels (CNS, colon, leukemia, lung, melanoma, ovarian and renal). The growth medium has a stable physiological pH of 7.4 in atmospheric CO_2 (0.04%) and derives its buffering capacity primarily from β-glycerophosphate. The basal growth medium was optimized to facilitate growth in atmospheric CO_2 by inclusion of (1) biotin, (2) L-asparagine, and (3) pyruvate and oxaloacetate for metabolic stimulation of intracellular CO_2 production. The new medium (PDRG-1) was shown to be suitable for use not only with the XTT tetrazolium assay, but also with other assays (e.g., sulforhodamine B protein assay) under evaluation for possible use in the NCI anticancer drug screen. Similar dose response curves for BCNU, VP-16, tamoxifen, mitomycin C, methotrexate and adriamycin were obtained against cell cultures maintained in PDRG-1 under ambient CO_2 or in RPMI-1640 under a 5% CO_2 environment.

Evaluation of Colorimetric Protein and Biomass Stains for Assaying In Vitro Drug Effects Upon Human Tumor Cell Lines. Twenty histological stains were evaluated for assay of in vitro anticancer activity in a high volume drug screen employing 60 human tumor cell lines. The stains included seven anionic dyes which bind to the basic amino acid residues of proteins and 13 cationic dyes which bind to the negative fixed charges of biological macromolecules (protein, RNA, DNA, GAGs). Optimal protocols were determined for each dye using TCA-fixed monolayers. Supramaximal concentration and staining time were determined by dose-response and kinetic analyses. Sulforhodamine B (SRB), bromophenol blue, orange G, chromotrope 2R, thionin, safranin O, phenosafranin, toluidine blue, azure A, and acridine orange all gave satisfactory ODs with monolayers grown in 96 well microtiter plates. SRB provided the best combination of stain intensity, signal to noise ratio, and linearity with cell number. It can detect about 1,000 cells at 560 nM, and 200-300 cells fluorescently. SRB is a bright pink anionic dye which in dilute acetic acid binds electrostatically to the basic amino acids of TCA-fixed cells. Based upon the extended testing of several thousand synthetic compounds against a panel of 60 human tumor cell lines, SRB appears to provide a reliable assay of anticancer drug activity.

Suitability of Multicellular Growth Units in Soft Agar Culture for Experimental Drug Evaluations and Morphologic Examinations. A variety of procedures have been described in the literature for the study of growth and drug sensitivity of human tumor material in soft agar culture with an emphasis on treatment of single cell suspensions. In the present study, the suitability of multicellular growth units (MGU) in soft agar culture for experimental drug evaluations was assessed using six human tumor cell lines. Cultures exposed continuously to each of six standard agents on days 1-7 were compared with replicate cultures treated on days 4-11. Growth was measured by image analysis in terms of MGU counts in multiple size categories and cumulative MGU volumes calculated on the basis of an algorithm for each cell line. Under conditions of assay, a given cell line exhibited similar IC 50 values for each agent (34/36 tests with >1 log difference) irrespective of treatment schedule, whereas multiple log differences were observed in percent survival depending upon treatment schedule (11/36 tests with a 2.05 to 3.46 log difference). In general, cultures containing MGU at the time of drug application resulted in more discriminating indices of drug effect than cultures containing single cells. MGU in soft agar culture can be generated for many human tumor cell lines: suitable growth rates and cloning efficiencies were observed for 13/21 lung, 12/13 colon, 10/11 CNS, 6/10 kidney, 13/13 melanoma, 4/7 ovary, and 5/5 leukemia cell lines. Results indicate that soft agar cultures (1) permit cultivation and quantification of MGU growth and drug sensitivity, (2) are well-suited for detection and isolation of clones which survive drug exposure, and (3) are amenable to a variety of histologic procedures.

Morphometric and Colorimetric Analysis of Human Tumor Cells in Culture. Previous studies have demonstrated the suitability of image analysis and tetrazolium staining to assess growth and drug sensitivity of human tumor cells cultivated in soft agar culture. In the present study, growth

profiles and drug sensitivity profiles were assessed by image analysis and by formazan colorimetry for purposes of comparison and to assess the suitability of colorimetric analysis of colony formation assays. Replicate cultures treated with INT or MTT exhibited nearly identical colony count and volume indices, as well as excellent correlation in colorimetric endpoints. Morphometric versus colorimetric assessment of the same cultures following DMSO extraction of PBS rinsed, dried soft agar cultures exhibited excellent linear correlation for both growth (r^2 ranging from 0.92 to 0.99, N = 18/each of 6 cell lines) and drug sensitivity (r^2 ranging from 0.88 to 0.94, N = 72 to 90 pairs/each of 6 cell lines). Detection ranges observed for colorimetric analysis and morphometric analysis were 1.3 - 2.2 log units and 3.3 - 5.4 log units, respectively. Results of the current investigation indicate that morphometric and colorimetric endpoints of soft agar culture remain stable for a period of two weeks following culture termination, and that each mode of analysis provides reliable measurements of growth and drug sensitivity of tumor cell lines cultivated in soft agar culture.

Morphologic Characterization of Human Carcinoma Cell Lines. Cell lines derived from human carcinomas are widely used for the study of human tumor biology and experimental therapeutics. The validity of these cell lines as "disease-oriented" models is dependent, in part, on the degree to which they retain the characteristics of their respective parent cancers. The present study was conducted to delineate the morphologic characteristics of candidate cell lines for an in vitro anticancer drug screen. Over 60 commonly used human tumor cell lines derived from cancers of the central nervous system, colon, lung, ovary, kidney, and from melanomas were analyzed. Cell lines were grown in monolayer culture and, when possible, in soft agar culture and in nude mice as xenografts. Histologic, histochemical and electron microscopic examinations were performed. In addition, immunocytochemical assays for a number of markers, including various intermediate filaments, carcinoembryonic antigen, as well as panels of tissue specific antigens, were performed. Based on these observations, at least 10 to 20% of the cell lines were diagnosed as undifferentiated carcinomas and could not be further classified. The majority of the other cell lines were very poorly differentiated, but retained sufficient morphologic or antigenic characteristics to allow classification consistent with that of the parent cancer, although representing the more poorly differentiated elements of such a tumor. Fewer than 20% of the cell lines were found to retain the spectrum of characteristics generally associated with the parent cancer type. These findings stress the importance of detailed characterization of cell lines to determine their suitability for particular experiments, and underscore the need for development of new cell lines which represent valid models for the in vitro study of human cancer.

Histopathologic, Biochemical, and Molecular Genetic Characterization of Four Newly Established Human Pulmonary Carcinoma Cell Lines. Four new human lung cancer cell lines (LCCL) have been developed in vitro from surgically resected primary pulmonary carcinoma tissue. These include: Hop 18 large cell undifferentiated carcinoma, Hop 19 and 62 adeno-

carcinomas, and Hop 27 large cell undifferentiated/small cell mixed carcinoma. These four new LCCL have been extensively characterized according to their individual histologic, cytologic, ultrastructural, biochemical and molecular genetic features and, when possible, these characteristics were compared with the original tumor specimens from which they were derived in order to confirm the authenticity of individual LCCL. Each LCCL was generally similar to the original tumor specimens from which they were derived, demonstrating that the LCCL have retained their original histopathologic, and biochemical characteristics. Individual LCCL were biochemically characterized for endogenous prostaglandin biosynthesis, as well as metabolism of different xenobiotics including 4-ipomeanol and benzo(a)pyrene. Expression of different cytochrome P-450 genes, a multi-drug resistant gene and glutathione-s-transferase gene were also studied in the four LCCL, as well as DNA fingerprint analyses using variable tandem repeat DNA probes. These biochemical and molecular genetic markers were effectively used to fingerprint the individual LCCL. These new, well characterized LCCL should be useful for study of the biology of human LC.

Expression of Different Cytochrome P450 Genes in Human Lung Cancer Cell Lines. Previous studies employing rodent models have suggested that cytochrome P450 (P450) enzyme activities are lost in neoplastic cells and that this loss of P450 activity is a marker for malignant phenotype (Cancer Research 44:5463, 1984). To date, however, virtually no information has been obtained on the expression of different P450 enzyme systems in human cancers. In the present studies, we have, therefore, investigated the expression of the P450 structural genes P450IA1(IA1), P450 IIE1 (IIE1) and P450 IVB1 (IVB1) in fresh normal human tissues, as well as in 23 human pulmonary carcinoma (PC) cell lines. The IIE1 and IVB1 genes were constitutively expressed in normal liver and lung tissue, respectively, while the IA1 gene was expressed only in normal lung tissue from cigarette smokers as a result of induction with cigarette smoke components. When individual PC lines were evaluated, constitutive (noninduced) IA1 mRNA levels were detected in 26% (6/23). However, when the cell lines were cultured in the presence of a IA1 inducer, 70% (16/23) expressed detectable IA1 mRNA, including 93% (14/15) of the nonsmall cell PC, but only 25% (2/8) of the small cell lines. Additionally, a 6-fold variation in induced IA1 mRNA levels was noted among the 23 PC. No detectable IIE1 or IVB1 mRNA was noted in any of the 23 cell lines studied. These data demonstrate that only the IA1 gene is expressed in human PC cell lines. Expression of specific P450 genes in human PC, especially the IA1 gene, might be important in the pathogenesis of lung cancer as well as providing a molecular genetic marker for this disease.

Expression of a New Cytochrome P450 Gene in Surgically Resected Normal Human Lung and Pulmonary Carcinoma Tissue. The cytochrome P450 (P450) monoxygenases are responsible for the metabolism of endogenous substrates such as steroids and prostaglandins, as well as the activation and/or detoxification of many xenobiotics including selected environmental carcinogens. Because P450s are potentially important in human lung homeostasis and pulmonary carcinogenesis, we investigated the expression

of a newly isolated P450 gene, designated P450L1(L1), in surgically-resected normal human lung (NL) and pulmonary carcinomas (PC) from 26 primary PC patients, including 15 current cigarette smokers (SM), eight former smokers who had not smoked for at least two months (FS), and three nonsmokers (NS). When NL was evaluated, L1 gene expression (GE) was present in 100% (20/20) irrespective of smoking history with a 6-fold variation in mRNA levels among different individuals. When PC were evaluated, only 47% (8/17) demonstrated L1 GE. Interestingly, only 1 PC from a SM expressed detectable L1 mRNA levels, while 66% (4/6) PC from ES and 100% (3/3) PC from NS demonstrated L1 GE. Furthermore, 85% (6/7) of the PC from FS or NS that expressed the gene were adenocarcinomas (including 4 bronchioloalveolar cell carcinomas). These results demonstrate that (1) constitutive L1 GE occurred in all NL regardless of smoking status, (2) a greatly decreased frequency of L1 GE was observed in PC from SM compared to those from NS and FS, suggesting a dysregulation of L1 GE in PC which is associated with cigarette smoking, and (3) L1 GE might potentially be important in NL homeostasis, as well as in the pathophysiology of human lung cancer.

Altered Regulation of Cytochrome P450IA1 Gene Expression in Established Human Pulmonary Carcinoma Cell Lines. Because of its potential significance in human lung cancer, we have, in the present studies, investigated the expression of the CYPIA1 structural gene in 24 established human lung cancer cell lines, including 15 nonsmall cell (8 adenocarcinomas, 3 large cell undifferentiated carcinomas, 2 bronchiolo-alveolar cell carcinomas and 2 squamous cell carcinomas) and nine small cell lung carcinomas. CYPIA1 mRNA was detected in 14 of 15 (93%) of the nonsmall cell lung carcinoma cell lines examined following 24 hr induction with benz(a)anthracene (BA) and in seven of 15 (47%) of the nonsmall cell lines cultured without an inducer in the medium. When the small cell lung cancer lines were evaluated for CYPIA1 gene expression, two of nine (22%) expressed detectable CYPIA1 mRNA in both BA-induced cell cultures, as well as constitutive (control) cultures. A positive correlation was noted between BA-induced CYPIA1 mRNA levels and the corresponding CYPIA1 enzyme activity expressed as absolute BA-induced enzyme activity ($r=0.915$; $p<0.01$; $N = 24$), further demonstrating that CYPIA1 mRNA expression reflects CYPIA1 enzyme activity in the individual cell lines. These observations represent the first known demonstration of constitutive (noninduced) CYPIA1 gene expression in human cells and suggest altered regulation of the CYPIA1 gene in selected lung cancer cell lines at the cis- and/or trans-regulatory level. Preliminary studies examining the possibility of gross alterations in the 5' cis-regulatory portions of the CYPIA1 gene within these cells were negative, possibly suggesting a trans-regulatory defect. These findings also demonstrate that the CYPIA1 gene is expressed in the majority (67%) of human lung cancers, further supporting a potential role for the CYPIA1 gene in human pulmonary carcinogenesis.

Evidence for Thromboxane Biosynthesis in Established Cell Lines Derived from Human Lung Adenocarcinomas. Thromboxane B_2 (TXB_2) is the stable nonenzymatic hydrolysis product of thromboxane A_2, a substance implicated in the initiation of the facilitative role of thrombocytes in the

metastatic process. TXB_2 was isolated from protein-free culture medium of cell lines Calu-3, Calu-6, A549, and A549/Asc-1, derived from human lung adenocarcinomas. TXB_2 and other 20-carbon fatty acid cyclooxygenase products synthesized from exogenous and endogenous arachidonic acid were identified by their characteristic retention indices and fragmentation of electron-capture derivatives of unlabeled and deuterium-labeled products during combined capillary gas chromatography-mass spectrometry. TXB_2 comprised 2 to 6% of 20-carbon fatty acid cyclooxygenase products biosynthesized from endogenous arachidonic acid in calcium ionophore A23187-stimulated Calu-6 and A549/Asc-1 cells and 16 to 25% of these products in Calu-3 and A549 cells. The addition of 10^{-5} M exogenous arachidonic acid to the cultured cells resulted in a 2- to 3-fold increase in TXB_2 and bisenoic prostanoid production with no significant alterations in the proportion of TXB_2 production. Prostaglandin E_2 and prostaglandin $F_{2\alpha}$, two prostanoids that can be formed either enzymatically or nonenzymatically from prostaglandin H_2, accounted for >75% of isolatable 20-carbon fatty acid cyclooxygenase products synthesized from endogenous and exogenous arachidonic acid.

Profiles of Prostaglandin Biosynthesis in Normal Lung and Tumor Tissue from Lung Cancer Patients. Prostaglandin (PG) biosynthetic profiles from endogenous arachidonic acid were determined by capillary gas chromatography-mass spectrometry in matched fresh normal lung (NL) and lung cancer (LC) tissue fragments obtained from 42 individual LC patients at the time of diagnostic thoracotomy. The histological diagnoses represented were squamous cell carcinoma (N = 20), adenocarcinoma (N = 7), small cell carcinoma (N = 4), mixed cell carcinoma (N = 2), bronchioloalveolar cell carcinoma (N = 2), large cell undifferentiated carcinoma (N = 3), bronchial carcinoid (N = 1), and metastatic tumors (N = 3). When PG biosynthesis was determined in NL tissue separately, low mean levels of PGE_2 and $PGF_{2\alpha}$ (<2 pmol/mg protein/15 min), intermediate levels of PGD_2 and 6-keto $PGF_{1\alpha}$ ($6KPGF_{1\alpha}$) (2-7 pmol/mg protein/15 min), and high levels of thromboxane B_2 (TXB_2) (>7 pmol/mg protein/15 min) were observed. There was no particular correlation with cigarette smoking history and PG biosynthesis in NL. When PG production in LC tissue was evaluated separately, high levels of PGE_2, $PGF_{2\alpha}$, and $6KPGF_{1\alpha}$, as well as TXB_2 and low levels of PGD_2 were noted. In addition, LC tissue from cigarette smokers demonstrated elevated levels of PGE_2, $6KPGF_{1\alpha}$, and TXB_2 when compared to current nonsmokers with LC ($P<0.05$ in all instances). Simultaneous comparison of PG production in matched LC and NL tissue from individual patients indicated increased biosynthesis of PGE_2 and $PGF_{2\alpha}$ and low levels of PGD_2 in LC compared to NL tissue ($P<0.05$ in all instances; paired, two-tailed, Student's t test). Individual comparison of PG biosynthesis according to LC histological cell type revealed that PGE_2 and $PGF_{2\alpha}$ were consistently elevated in all four common primary LC histological cell types, the only exception being large cell undifferentiated carcinoma. Interestingly, this latter LC histological cell type presented a unique profile with lower levels of PGE_2 and PGD_2 in LC than in NL tissue ($P<0.05$ in both instances). In addition, the biosynthesis of all five PGs studied was consistently higher in primary than metastatic adenocarcinomas of the lung ($P<0.05$ in all instances). No differences were observed in NL and LC tissue for the major LC histo-

logical cell types when PGD_2, or $6KPGF_{1\alpha}$ biosyntheses were compared. These findings indicate that the profiles of PG biosynthesis in LC and NL tissue from individual patients may differ substantially. These differences may reflect, in part, contributions to the PG biosynthetic profile unique to malignant cells.

Profiles of Prostaglandin Biosynthesis in Sixteen Established Cell Lines Derived from Human Lung, Colon, Prostate, and Ovarian Tumors. The profiles of prostanoid biosynthesis from endogenous arachidonic acid in 16 established cell lines derived from four histological classes of human carcinomas were determined by capillary gas chromatography-mass spectrometry. Detectable quantities of prostanoids were isolated from the culture medium of cell lines representative of the different histological classes of human tumors: colorectal adenocarcinomas (1 of 3 cell lines); ovarian adenocarcinomas (1 of 3 cell lines); prostate adenocarcinomas (0 of 2 cell lines); non-small cell carcinomas of the lung (4 of 5 cell lines); and small cell carcinomas of the lung (0 of 3 three cell lines). Prostaglandins E_2 and $F_{2\alpha}$ were the only prostanoids synthesized in detectable quantities. Prostaglandin E_2 biosynthesis (mean $\pm$ SD), pmol/10^6 cells, $\underline{N} = 4$) in cell lines exhibiting positive prostaglandin H synthase activity was: LoVo (colorectal adenocarcinoma, 0.4 ± 0.1); A2780 (ovarian adenocarcinoma, 1.3 ± 0.3); NCI-H322 (bronchioloalveolar cell carcinoma, 8.4 ± 3.1); NCI-H358 (bronchioloalveolar cell carcinoma, 7.8 ± 2.4); EKVX (adenocarcinoma of the lung, 21.3 ± 5.5); and A427 (large cell undifferentiated carcinoma of the lung, 12.6 ± 2.8). Prostaglandin $F_{2\alpha}$ production (pmol/10^6 cells $\pm$ SD) was: LoVo (0.3 ± 0.1); NCI-H322 (0.6 ± 0.2); NCI-H358 (0.4 ± 0.1); EKVX (1.8 ± 0.4); and A427 (11.1 ± 3.1). These findings suggest that, within certain limitations, cultured tumor cells provide simplified experimental systems for determination of prostaglandin biosynthetic characteristics of human tumors, and that prostanoid biosynthesis may be particularly characteristic of certain non-small cell carcinomas of the lung.

Evidence for Prostanoid Biosynthesis as a Biochemical Feature of Certain Subclasses of Non-small Cell Carcinomas of the Lung as Determined in Established Cell Lines Derived from Human Lung Tumors. Detectable levels ($\geq$0.2 pmol/10^6 cells) of one or more prostanoid species resultant to calcium ionophore A23187-induced biosynthesis from endogenous arachidonic acid were distributed in 28 cell lines derived from different histological classes of lung tumors as follows: large cell undifferentiated carcinoma (3 of 3 cell lines); adenosquamous carcinoma (1 of 2 cell lines); squamous cell carcinoma (0 of 2 cell lines); adenocarcinoma (9 of 10 cell lines); bronchioloalveolar cell carcinoma (2 of 2 cell lines); and small cell carcinoma (1 of 9 cell lines). Using the mean levels of $9\alpha,11\beta$-prostaglandin F_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2, thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ as an index of prostaglandin H (PGH) synthase activity, the distribution in cell lines representative of the different histological classes of human lung tumors exhibiting PGH synthase activity exceeding mean values $\geq$2 pmol/10^6 cells was as follows: large cell undifferentiated carcinoma (3 of 3 cell lines); adenosquamous

carcinoma (1 of 2 cell lines); adenocarcinoma (8 of 10 cell lines); bronchioloalveolar cell carcinoma (2 of 2 cell lines); and small cell carcinoma (0 of 9 cell lines). Three different prostanoid species accumulated to mean levels $\geq$2 pmol/10^6 cells. Prostaglandin E_2 levels exceeded 2 pmol/10^6 cells in 14 of the 16 cell lines in which this prostanoid accumulated to detectable levels. Cumulative levels of prostaglandin $F_{2\alpha}$ exceeded 2 pmol/10^6 cells in 9 of the 15 cell lines in which prostaglandin $F_{2\alpha}$ reached detectable levels. Detectable levels of thromboxane B_2 were observed in five cell lines with thromboxane B_2 accumulation exceeding 2 pmol/10^6 cells in two of the five cell lines. 9α,11β-prostaglandin F_2 and 6-keto-prostaglandin F_1 accumulated to detectable levels in the culture medium of one cell line, while prostaglandin D_2 accumulation to detectable levels was observed in two cell lines. Stimulation of cultured human lung tumor cells exhibiting PGH synthase activity $\geq$2 pmol/10^6 cells in the presence of 10^{-5} M exogenous arachidonic acid resulted in a 2- to 4-fold increase in the accumulation of individual prostanoids, while the inclusion of a 10^{-5} M exogenous concentration of arachidonic acid failed to stimulate detectable prostanoid production in human lung tumor cells in which PGH synthase activity was not previously expressed. The predominance of PGH synthase activity in cell lines derived from human non-small cell carcinomas of the lung suggest that prostanoid biosynthesis may be characteristic of tumor cells comprising certain histological subclasses of human non-small cell carcinomas of the lung, particularly adenocarcinoma, bronchioloalveolar cell carcinoma, large cell undifferentiated carcinoma, and possibly adenosquamous carcinoma.

Comparison of Intrapulmonary, Percutaneous Intrathoracic, and Subcutaneous Models for the Propagation of Human Pulmonary and Nonpulmonary Cancer Cell Lines in Athymic Nude Mice. The propagation efficiencies, growth patterns, histological appearances, and roentgenographic demonstration of tumors derived from six continuous human pulmonary tumor cell lines implanted intrathoracically (i.t.) and intrabronchially (i.b.) were compared with the conventional s.c. implantation method at three different tumor cell inocula (N = 184, i.b.; N = 185, i.t.; N = 180, s.c.). A tumor-related mortality of 100% was noted when the six different human lung tumor cell lines, including A549 adenocarcinoma, NCI-H125 adenosquamous carcinoma, NCI-H460 large cell undifferentiated carcinoma, NCI-H69 small cell carcinoma, and NCI-H358 and NCI-H322 bronchioloalveolar cell carcinomas, were implanted i.b. at a 1.0 x 10^6 tumor cell inoculum. A similar (92%) tumor-related mortality was observed when these same lung tumor cell lines were implanted i.t. at a 1.0 x 10^6 tumor cell inoculum (P>0.10), whereas minimal (5%) tumor-related mortality was noted when cells from the six different cell lines were implanted s.c. (P>0.001). In addition, a dose-dependent, tumor-related mortality was noted for either i.t. or i.b. implantation when lower (1.0 x 10^5 or 1.0 x 10^4) tumor cell inocula were employed. Histological characteristics and growth patterns of tumors propagated employing the three implantation techniques were closely comparable for all three propagation methods and, in all instances, histological appearances of the tumors were representative of the current tumor cell lines from which they were derived. Approximately 30% of the lung tumors

propagated i.t. grew in the chest wall and/or in the lung parenchyma, as well as in the pleural space. In contrast, tumors propagated i.b. grew predominantly in the lung parenchyma. When five nonpulmonary human tumor cell lines (including U251 glioblastoma, LOX amelamontic melanoma, HT-29 colon adenocarcinoma, OVCAR 3 ovarian adenocarcinma, and adriamycin-resistant MCF-7 breast adenocarcinoma) were propagated i.b. or i.t., there was considerable site-specific variability in tumor-related mortality depending on the tumor type. These data demonstrate that both the i.b. and i.t. models should be useful for the in vivo propagation and study of certain human pulmonary and nonpulmonary carcinomas, as well as being advantageous for future studies of cancer biology and developmental therapeutics.

Extrapulmonary, Tissue-specific Metastasis Formation in Nude Mice Injected with FEMX-I Human Melanoma Cells. FEMX-I human malignant melanoma cells, originating from a lymph node metastasis in a patient, uniquely and selectively produced extrapulmonary metastases after i.v. injection of cells prepared from xenografts into adult, nude mice. After a lag time of approximately 50 days, metastases were observed in s.c. sites at the back and front of the neck, and in axilla and inguinal regions. Tumor colony formation in lungs were never detected. The interscapular tumors showed a close relationship to brown fat, partly infiltrating this tissue, whereas the other s.c. tumors seemed to be localized to lymph nodes. Mesenterial and mediastinal lymph node metastases were frequently found, together with retroperitoneal tumors along the spine. The normal cells of the adrenal medulla were often replaced by melanoma cells, whereas the cortical tissue was not affected. The conclusion that FEMX-I cells possess an inherent ability for tissue-specific metastasis formation is supported by the metastatic pattern seen after i.p. and intrasplenic injection, as well as after inoculation of the cells in the footpads of the mice. The relatively slowly growing FEMX-I tumors showed the same differentiated morphology as the patient's tumor, independent of the site of growth and the number of passages in the animals. The FEMX-I tumor was easily established as a cell line in vitro. Such cells showed a strongly reduced metastatic capacity, indicating that the in vitro growth conditions had induced alterations in the FEMX-I cells influencing their ability to form site-specific metastases, changes that were shown to be reversible. It is suggested that structures on the surface of the tumor cells, as well as growth factors in the host tissues, may be of importance for the observed tissue specificity. The FEMX-I melanoma, which, as a human tumor in nude mice, has a unique metastatic pattern, offers possibilities for investigating mechanisms involved in site-specific metastasis formation, as well as for testing effects of antimetastatic, chemotherapeutic, and immunotherapeutic agents against human extrapulmonary micro- and macrometastases.

Flavone-8-acetic Acid Augments Systemic Natural Killer Cell Activity and Synergizes with IL-2 for Treatment of Murine Renal Cancer. The investigational drug flavone-8-acetic acid (FAA) potently augments NK activity in the spleen, liver, lungs, and peritoneum in a dose-dependent manner after i.v. or i.p. administration. Augmented NK activity peaks by

24 hours after FAA injection and returns to normal after six days. Combined treatment of established murine renal cancer with FAA and rIL-2 results in up to 80% long-term survival, whereas FAA or rIL-2 alone were unable to induce any long-term survivors. The optimal dose of rIL-2 required for use with FAA was in the range of 10,000 to 30,000 U/day. Further studies demonstrated that the regimen of FAA plus rIL-2 administration that was effective in treating established murine renal cancer also induced a more potent augmentation of NK activity than did either FAA or rIL-2 alone. Subsequent studies revealed that the therapeutic effectiveness of FAA plus rIL-2 was significantly reduced when tumor-bearing mice were treated with antiasialo GM_1 serum. These results are consistent with a role for augmented NK activity in the therapeutic effects of FAA plus rIL-2 against murine renal cancer. In addition, these studies demonstrate that FAA and rIL-2 is a useful approach for cancer treatment in that subtoxic doses of rIL-2 can be used and significant antitumor efficacy occurs even without accompanying adoptive immunotherapy.

Mechanistic Assays for Natural Product Anti-HIV Activity. The objective of this project is to develop rapid quantitative microassays to study the mechanisms of action of compounds with anti-HIV activity isolated from natural products. Microculture procedures were utilized to define the various stages of HIV infection in T-lymphoblastoid cells. Quantitative readouts of these assays included several novel colorimetric, enzymatic, and fluorometric endpoints. Interactive laser cytometry was also used to study the effects of anti-HIV compounds at the single cell level. In addition to developing quantitative tetrazolium and fluorometric assays for the overall cytopathic effects of HIV, microculture assays measuring the effects of compounds on virus/target cell interactions, reverse transcriptase and infectious viral release were also developed. Quantitative measurement of viral antigen expression at the single cell level was accomplished using immunocytofluorometric methods. Antiviral compounds with known mechanisms of action and comparative methodologies were used for assay validation. In continuing investigations, we are attempting to determine where, in the life cycle of HIV, natural products exert their activity. Understanding the mechanisms of action of antiviral compounds will aid in the selection and prioritization of novel anti-HIV agents for clinical trials.

Temporal Expression of HIV-associated Antigens Detected by Quantitative Immunofluorescence Using Interactive Laser Cytometry. We are investigating the feasibility of quantitative immunofluorescence techniques for rapid, large-scale screening assays of possible anti-HIV compounds. An Anchored Cell Analysis and Sorting (ACAS) interactive laser cytometer (Meridian Instruments, Okemos, MI) gave quantitative immunofluorescence data on the expression of the HIV-1 envelope glycoprotein, gp120, and the HIV-1 core protein, p24. The ACAS permitted quantitative fluorescence detection and evaluation not possible with conventional fluorescence microscopy. The expression of viral-derived gp120 and p24 appear to be early events subsequent to HIV-1 infection. In the case of gp120, these are important molecules associated with the tissue selectivity and cytopathic effects (i.e., syncytium formation) of

HIV-1 infection. Therefore, these markers were particularly appropriate for our initial immunofluorescence analyses. In indirect immunofluorescence assays using a panel of anti- gp120 and p24 monoclonal antibodies, we used the ACAS system to analyze the fluorescence signals generated by cell lines (i.e., H9 and CEM) infected with the rf strain of the Haitian variant of HIV-1 (HIV-1rf). Since this method proved to be particularly sensitive in detecting gp120 and p24 expression, the antibodies were used to examine expression of gp120 and p24 at several different time points after infection with HIV-1rf. This methodological approach permitted (1) early and very sensitive non-subjective and statistically based distinctions in the expression of gp120 and p24 by HIV-infected target cells, (2) evaluation of additional quantitative morphological information (i.e., mean fluorescence intensity and cell size) not generally possible with other fluorescence detection systems, and (3) such analyses on target cells stained after fixation (an important safety consideration).

AIDS-antiviral Sulfolipids from Cyanobacteria (Blue Green Algae). A recently developed tetrazolium-based microculture assay was used to screen extracts of cultured cyanobacteria for inhibition of the cytopathic effects of the human immunodefic immunodeficiency virus (HIV), which is implicated as a causative agent of AIDS (acquired immune deficiency syndrome). A number of extracts were found to be remarkably active against the AIDS virus. A new class of HIV-inhibitory compounds, the sulfonic acid-containing glycolipids, was discovered through the use of the microculture assay to guide the fractionation and purification process. The pure compounds were active against HIV in cultured human lymphoblastoid CEM, MT-2, LDV-7 and C3-44 cell lines in the tetrazolium assay, as well as p24 viral protein and syncytia formation assays.

Publications:

McLemore TL, Eggleston JC, Shoemaker RH, Abbott BJ, Bohlman ME, Liu MC, Fine DL, Mayo JG, Boyd MR. Comparison of intrapulmonary, percutaneous intrathoracic, and subcutaneous models for the propagation of human pulmonary and nonpulmonary cancer cell lines in athymic nude mice, Cancer Res 1988;48:2880-6.

Fodstad O, Kjonniksen I, Aamdal S, Nesland JM, Boyd MR, Pihl A. Extrapulmonary, tissue-specific metastasis formation in nude mice injected with FEMX-1 human melanoma cells, Cancer Res 1988;48:4382-8.

Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/ formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines, Cancer Res 1988;48:4827-33.

Wiltrout RH, Boyd MR, Back TC, Salup RR, Arthur JA, Hornung RL. Flavone-8-acetic acid augments systemic natural killer cell activity and synergizes with IL-2 for treatment of murine renal cancer, J Immunol 1988;140:3261-5.

Shoemaker RH, McLemore TL, Abbott BJ, Fine DL, Gorelik E, Mayo JG, Fodstad O, Boyd MR. Human tumor xenograft models for use with an in vitro-based, disease-oriented antitumor drug screening program. In: Pinedo HM, Peckham MJ, Winograd B, eds. European school of oncology monograph: human tumor xenografts in anticancer drug development. Berlin: Springer-Verlag, 1988;115-20.

Hubbard WC, Alley MC, McLemore TL, Boyd MR. Evidence for thromboxane biosynthesis in established cell lines derived from human lung adenocarcinmas, Cancer Res 1988;48:2674-7.

McLemore TL, Hubbard WC, Litterst CL, Liu MC, Miller S, McMahon NA, Eggleston JC, Boyd MR. Profiles of prostaglandin biosynthesis in normal lung and tumor tissue from lung cancer patients, Cancer Res 1988;48:3140-7.

Hubbard WC, Alley MC, McLemore TL, Boyd MR. Profiles of prostaglandin biosynthesis in sixteen established cell lines derived from human lung, colon, prostate, and ovarian tumors, Cancer Res 1988;48:4770-5.

Paull KD, Shoemaker RH, Boyd MR, Parsons JL, Risbood PA, Barbera WA, Sharma MN, Baker DC, Hand E, Scudiero DA, Monks A, Alley MC, Grote M. The synthesis of XTT: a new tetrazolium reagent that is bioreducible to a water-soluble formazan, J Hetero Chem 1988;25:911-4.

Boyd MR. Strategies for the identification of new agents for the treatment of AIDS: a national program to facilitate the discovery and preclinical development of new drug candidates for clinical evaluation. In: DeVita VT Jr, Hellman S, Rosenberg SA, eds. AIDS: etiology, diagnosis, treatment, and prevention, 2nd ed. Philadelphia: JB Lippincott, 1988;305-19.

Vince R, Hua M, Brownell J, Daluge S, Lee F, Shannon WM, Lavelle GC, Qualls J, Weislow OS, Kiser R, Canonico PG, Schultz RH, Narayanan VL, Mayo JG, Shoemaker RH, Boyd MR. Potent and selective activity of a new carbocyclic nucleoside analog (carbovir: NSC 614846) against human immunodeficiency virus in vitro, Biochem Biophys Res Commun 1988;156:1046-53.

Boyd MR, Shoemaker RH, McLemore TL, Johnston MR, Alley MC, Scudiero DA, Monks A, Fine DL, Mayo JG, Chabner BA. New drug development. In: Roth JA, Ruckdeschel JC, Weisenburger TH, eds. Thoracic oncology. WB Saunders, 1989;711-21.

Hubbard WC, Alley MC, Gray GN, Green KC, McLemore TL, Boyd MR. Evidence for prostanoid biosynthesis as a biochemical feature of certain subclasses of non-small cell carcinomas of the lung as determined in established cell lines derived from human lung tumors, Cancer Res 1989;48:826-32.

Weislow OW, Kiser R, Fine DL, Bader J, Shoemaker RH, Boyd MR. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity, JNCI 1989;81:577-86.

Boyd MR. Status of implementation of the NCI human tumor cell line in vitro primary drug screen, Proc Am Assoc Cancer Res 1989;30:652-4.

Paull KD, Hodes L, Shoemaker RH, Monks A, Scudiero DA, Rubinstein L, Plowman J, Boyd MR. Approaches to the display and analysis of patterns of differential activity of drugs against a panel of human tumor cell lines I. development of the "mean graph" and the "compare" algorithm, JNCI 1989; in press.

Christian MC, Wittes RE, Leyland-Jones B, McLemore TL, Smith AC, Grieshaber CK, Chabner BA, Boyd MR. 4-Ipomeanol: a novel investigational new drug for lung cancer, JNCI 1989; in press.

McLemore TL, Adelberg S, Czerwinski M, Hubbard WC, Yu SJ, Storeng R, Wood T, Hines R, Boyd MR. Altered regulation of cytochrome P450IA1 gene expression in established human pulmonary carcinoma cell lines, JNCI 1989; in press.

Gustafson KR, Cardellina JH Jr, Fuller RW, Weislow OS, Kiser RF, Snader KM, Patterson GML, Boyd MR. AIDS-antiviral sulfolipids from cyanobacteria (blue green algae), JNCI 1989; in press.

McMahon JB, del Campo AA, Prather TR, Boyajian SL, Grady BA, Jones GM, Plopper C, Boyd MR. Factors affecting proliferation of alveolar type II cells in culture, Exptl Cell Res 1989; in press.

Alley MC, Hursey ML, Pacula-Cox CM, Boyd MR. Morphometric and colorimetric analyses of human tumor cells in soft agar culture, Cancer Res 1989; in press.

McLemore TL, Abbott BJ, Mayo JG, Boyd MR. Development and application of new orthotopic in vivo models for use in the U.S. National Cancer Institute's drug screening program. In: Wu B, Zheng J, eds. 6th international workshop on immunodeficient animals in biomedical research. Basel: S Karger, 1989; in press.

ANNUAL REPORT OF THE DRUG SYNTHESIS & CHEMISTRY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The fundamental responsibility of the Drug Synthesis and Chemistry Branch (DS&CB) is the discovery of novel leads for drug development against cancer and AIDS. This is achieved through a variety of program activities; namely, acquisition of a large number of synthetic compounds and natural products of diverse biological and structural types through the development and maintenance of an extensive network of scientific liaison on a world-wide basis; lead optimization through the synthesis of congeners and prodrugs; radiolabelled syntheses; task order resyntheses; storage, inventory, and distribution; and computer modeling. Over the past year, nearly 6000 new compounds, both natural and synthetic in origin, were acquired for screening through ongoing scientific liaison. A base of chemical and biological data has been accumulated on some 450,000 compounds. Most of these are stored in a DS&CB repository, which manages the receipt and shipment to screening laboratories. The DS&CB plays a key role in operation of the Acquisition Input Committee, which manages the flow of compounds through the NCI Screens. About 200 pure compounds monthly are being submitted to the AIDS screen. A tracking system of 12 check points was recently devised in cooperation with the Information Technology Branch and results on 90% of the compounds tested vs AIDS are now returned to suppliers within 4 months of submission. Identification of structure-activity relationships by computer-assisted analysis of test results is well-established within DS&CB.

Activity leads are optimized through the synthesis of prodrugs and congeners. Other projects are specifically for resynthesis (of compounds unavailable from the literature, and for scale-up of lead compounds) and for radiolabelled synthesis. Current efforts include the synthesis of active ATA polymers vs AIDS, nonclassical isosteres as bombesin antagonists vs cancer, and protein kinase inhibitors related to erbstatin vs cancer. Carbovir, described a year ago as a new lead vs AIDS, has now been licensed to a drug company and is under commercial development. The first total synthesis of the novel anti-AIDS product from cyanobacteria was recently completed. A novel and highly promising lead against AIDS was recently identified in a series of 450 synthetic compounds submitted by a non-pharmaceutical chemical company; this compound is being pursued actively. Another program commitment is to collaborate with DTP intramural scientists by synthesis of compounds for special projects; current examples are a series of anticancer N-mustards and CoQ analogs.

Over the past year, DS&CB has assumed new responsibilities for acquisition of natural products.

The DS&CB is staffed with nine professionals and two clerical personnel. The contracts managed by DS&CB are outlined in Table 1:

TABLE I

CONTRACTS - FY 89

Contractor	Investigator	Contract No.
Alabama, University of	Baker	N01-CM-87267
ERCI Facilities Service Corp.	Groover	N01-CM-73721
Georgia Technology Res. Center	Zalkow	N01-CM-87269
Illinois, University of	Farnsworth	N01-CM-87226
Japanese Foundation	Tsukagoshi	N01-CM-36011
Moravek Biochemicals, Inc.	Moravek (SBIR)	N44-CM-77830
Naprotech Research Lab	Che	N01-CM-97562
New Mexico, State University of	Guziec	N01-CM-87278
New York, State University of	Anderson	N01-CM-67698
New York, State University of	Anderson	N01-CM-87216
Purdue Research Foundation	Cushman	N01-CM-67699
Purdue Research Foundation	Cushman	N01-CM-87268
Research Triangle Institute	Kepler	N01-CM-67703
Research Triangle Institute	Kepler	N01-CM-87227
Southern Research Institute	Hayes	N01-CM-87229
Starks Associates, Inc.	D. Starks	N01-CM-67978
Starks Associates, Inc.	Risbood	N01-CM-87231
Starks Associates, Inc.	Perchonock	N01-CM-97569
Z, Inc.	Sobers	N01-CM-73720

Master Agreements (Task Order) Contracts:

Alabama, University of	Baker	N01-CM-67971
Battelle Memorial Institute	Fentiman	N01-CM-97612
Darmouth College	Curphey	N01-CM-67976
H.G. Pars Pharmaceutical Labs.	Pars	N01-CM-67972
Metafluor, Inc.	Hale	N01-CM-97614
New Mexico, State University of	Guziec	N01-CM-67974
Polysciences, Inc.	Parasaran	N01-CM-67977
Raylo Chemicals, Ltd.	Lee	N01-CM-67975
Research Triangle Institute	Seltzman	N01-CM-67970
Ricerca, Inc.	Buchman	N01-CM-97613
South Florida, University of	Owen	N01-CM-67973
Southern Research Institute	Temple	N01-CM-67968
SRI International	Tanga	N01-CM-67969
Starks Associates	Hsiao	N01-CM-67978
Synthetech, Inc.	Knutson	N01-CM-97616

PUBLICATIONS:

Silverton SV, Quinn FR, Haugwitz RD, Todaro LJ. Structures of two dideoxynucleosides: 2',3'-dideoxyadenosine, and 2',3'dideoxy-cytidine, Acta Cryst 1988;44:321.

Choi H-K, Terzis A, Stevens RC, Bau R, Haugwitz RD, Narayanan VL, Wolpert-DeFilippes M. Reaction products of a new anticancer agent, Pt(IV) (cyclohexyldiamine)Cl_4, with guanosine and 9-methylguanine: first crystal structures of Pt(cyclohexyldiamine) complexes with a nucleobase and a nucleoside, Biochem Biophys Res Commun 1988;156:1120-1124.

Deutsch HM, Glinski JA, Hernandez M, Haugwitz RD, Narayanan VL, Suffness M, Zalkow LH. Synthesis of congeners and prodrugs. 3. Water-soluble prodrugs of taxol with potent antitumor activity, J Med Chem 1989;32:788-792.

Hodes L. Clustering a large number of compounds. I. Establishing the method on an initial sample, J Chem Info Comput Sci 1989;29: 66-71.

Plowman J. Paull KD, Attassi G, Harrison Jr. SD, Dykes DJ, Kabbe HJ, Narayanan VL, Yoder OC. Preclinical antitumor activity of batracylin (NSC 320846), Investigational New Drugs 1988;6: 147-153.

Plowman J, Harrison Jr. SD, Dykes DJ, Paull KD, Narayanan VL, Tobol HK, Martin J, Griswold Jr. DP. Preclinical antitumor activity of an α-picoline derivative, penclomedine (NSC 338720), on human and murine tumors, Cancer Res 1989;49:1909-1915.

Vince R, Hua M, Brownell J, Daluge S, Lee F, Shannon WM, Lavelle GC, Qualls J, Weislow OS, Kiser R, Canonico PG, Schultz RJ, Narayanan VL, Mayo JG, Shoemaker RH, Boyd MR. Potent and selective activity of a new carbocyclic nucleoside analog (carbovir: NSC 614846) against human immunodeficiency virus *in vitro*, Biochem Biophys Res Commun 1988;156:1046-1053.

Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, Boyd MR. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm, J Natl Cancer Inst. In press.

Chapter in a Book

Hodes L. Computer-aided selection for large-scale screening. In: Kennewell PD, ed. Comprehensive Medicinal Chemistry, Chapter 3.3, Vol. 1, general introduction. Oxford: Pergamon Press. In Press.

Narayanan VL. Development of new chemotherapeutic agents. In: Magrath I, ed. New directions in cancer treatment. New York: Springer-Verlag. In press

Nasr M, Paull KD, Narayanan VL. Computer assisted structure-anticancer activity correlations of organotin compounds. In: J J Zukerman, ed. Tin and malignant cell growth. Boca Raton: CRC Press, 1988;212-226.

PATENTS:

Haugwitz RD, Narayanan VL, Zalkow LH, Deutsch HM, Gelbaum L. US Patent 4,803,202: Substituted N-Methyl Derivatives of Mitindomide, February 7, 1989.

ANNUAL REPORT OF THE NATURAL PRODUCTS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 - September 30, 1989

The Natural Products Branch (NPB) is responsible for acquisition, isolation, structure determination, and testing of compounds from microbial, plant and animal sources in order to discover new leads for further development in the NCI programs for both anticancer and anti-AIDS drugs.

The major program areas of the Branch are: (1) acquisition of crude biological materials of plant, marine, and microbial origin for the DTP screening programs; and (2) coordination of research directed toward isolation of new agents from active extracts. The development by DTP of sensitive in vitro anticancer and AIDS antiviral screens has resulted in a resurgence of interest in natural products as sources of potentially selective antitumor and anti-HIV agents, and the focus of Branch programs is now the discovery of new leads from natural sources for development into potential anticancer and AIDS antiviral drugs. Contracts for the collection of plants from tropical rain forest areas and shallow-water marine organisms from the Indo-Pacific region have been in progress since September 1986, as has a contract for the cultivation of cyanobacteria. A contract for the cultivation of fungi was completed in December, 1988, and one for the cultivation of marine protozoa was awarded in March, 1989. Initiatives for the cultivation of marine anaerobic bacteria and unusual fungi are currently under consideration.

An important aspect of the Branch's efforts during FY '89 has been the coordination of the various stages of the DTP program for the discovery of novel anti-HIV agents from natural sources, including acquisition of raw materials, extraction, extract formulation, screening, and isolation of agents from active extracts. Over 6,000 extracts have been tested for AIDS antiviral activity, and a number of extracts of plant, marine and microbial origin have been found to possess significant in vitro anti-HIV activity. A novel class of in vitro active products has been isolated from cyanobacterial extracts by the DTP intramural isolations chemistry group, and work is proceeding on the fractionation and purification of other active extracts.

Organization and Staffing

The Branch is organized into three functional segments which are: (1) discovery of new agents from fermentation derived extracts, (2) discovery of new agents from plant extracts, and (4) discovery of new agents from extracts of marine organisms. Many tasks require

interaction between these segments, and the Branch personnel are assigned duties in whichever of the areas requires their expertise depending on changing program needs. The present full time staff consists of three professionals and one secretary. The contracts managed by the Branch are outlined in Table 1.

Table 1.

Natural Products Branch Contracts

Contractor	Investigator	RFP No.	Contract Program Area
		CANCER	
Harbor Branch Oceanographic Institution	Pomponi	NCI-CM8-7292	Shallow Water Marine Collection
Univ. of Illinois at Chicago	Soejarto	NCI-CM6-7925	Plant Collection (Asia)
Missouri Botanical Garden	Forero	NCI-CM6-7923	Plant Collection (Africa)
New York Botanical Garden	Balick/Daly	NCI-CM6-7924	Plant Collection (South America)
Univ. of Hawaii at Manoa	Patterson	NCI-CM6-7745	Cultivation of Cyanobacteria
Martek Corporation	Cox	NCI-CM9-7615	Marine Protozoa
		AIDS	
Univ. of Hawaii at Manoa	Sagawa	NCI-CM8-7282	Plant Recollections
World Botanical Associates	Spjut	NCI-CM8-7281	Plant Recollections
Battelle, Columbus Division	Blake	NCI-CM8-7233	Shallow Water Marine Recollections

Battelle, Columbus Division	Blake	NCI-CM8-7280	Deep Water Marine Recollections
Martek Corporation	Cox	NCI-CM8-7283	Regrowth of Phototrophic Microorganisms

Microbial Cultivation Program

To date, many of the natural products which have progressed to advanced preclinical and clinical development have been isolated from land-based bacteria, actinomycetes and fungi. DTP has now developed programs which expand beyond the microorganisms traditionally studied in the pharmaceutical industry, and is taking a leadership position in cultivating novel source organisms to provide extracts for screening. A number of extracts from the recently completed fungal cultivation contract with the University of Connecticut and the ongoing cyanobacterial (blue-green algae) contract with University of Hawaii have exhibited preliminary *in vitro* AIDS antiviral activity, and a novel class of products showing *in vitro* antiviral activity has recently been isolated from several cyanobacterial extracts. A contract for the cultivation and extraction of marine protozoa was awarded to Martek Corporation in March, 1989, and initiatives in the cultivation of marine anaerobic bacteria and unusual fungi are currently being considered.

Plant Program

Three major contracts for the collection of over 20,000 plant samples from the tropical rain forest regions of Africa, Madagascar, Central and South America, and South East Asia were awarded in September, 1986 for a five year period. Good progress has been made in the collection of a wide variety of taxa, and some contact has been made with shamans and traditional healers in certain areas who are helping in the collection of medicinal plants. To date, over 10,000 samples have been received of which over 4,033 have been extracted to yield 8,066 extracts.

Master Agreement Contracts for the recollection of plants for the isolation of potential AIDS antiviral agents have been awarded to University of Hawaii at Manoa and World Botanical Associates.

Marine Program

Two major contracts were initiated September 30, 1986 for the collection and documentation of 5,000 shallow-water and 5,000 deep-water organisms over a five year period. The collections include a wide variety of taxa, selected to represent the greatest possible chemical, taxonomic and environmental diversity, and priority is given to taxa known to contain biologically-active metabolites. The Shallow Water collection contract is meeting its goal of more than 1,000 specimens per year and 1,797 are on hand in the FCRF Repository. The Deep Water Marine program was terminated after one

year due to escalating costs, difficulties in scheduling ship time and insufficient amounts of samples collected. Extraction protocols for marine organisms have been finalized, and over 150 extracts have been prepared thus far. Master Agreement Contracts for the recollection of shallow and deep-water marine organisms for the isolation of potential AIDS antiviral agents have been awarded to the Battelle Memorial Institute, Columbus Division.

Deep Water collection may become more efficient in the next few years as technology for ROVs (remotely operated vehicles) improves and these can be used in collection operations instead of the much more expensive manned submersibles. NCI may therefore wish to restart this project at a later time depending on technological advances and the results obtained on screening the 804 Deep Water samples collected to date.

Natural Products Extraction Laboratory and Repository

A natural products extraction laboratory and repository is operating at the Frederick Cancer Research Facility, and all plant and marine organism samples collected under contract are sent to this facility. Following logging in of the raw material samples by repository personnel, the samples are extracted with an organic solvent and water according to standard protocols which have been specially devised by laboratory and DTP staff to achieve optimum extraction efficiency. These extracts, and those submitted by the contractors responsible for the microbial cultivation projects, are stored in the repository at -20°C to await testing in the human cancer cell line and HIV screens; samples of these extracts will also be held for testing in later screens as they are developed. A natural products computer support system has been developed which records the progress of each sample from receipt of the raw material, through the extraction process, to deposit of the extracts in the low-temperature repository. Reference to the various databases comprising this system enable the retrieval of detailed information concerning every organism extracted and the nature and repository storage location of each extract sample.

The status of natural products acquisition and screening is summarized in Table 2.

Table 2.

Natural Products Acquisitions

September, 1986 - May, 1989

	Organisms Received	Organisms Extracted	Extracts Available	Extracts Tested for Anti-HIV Activity
Deep Sea Marine Organisms	804	18	36	36
Shallow Water Marine Organisms	1797	10	131	131
Cyanobacteria	537	537	1074	497
Fungi	1101	1101	13212	1089
Terrestrial Plants	10,000	4033	8066	3397
Marine Plants	167	167	323	323
Lichens	517	517	1033	1033

Accomplishments

The major program for the discovery of new leads for development into anticancer and AIDS antiviral agents is proceeding well. Significant preliminary *in vitro* anti-HIV activity has been detected in a number of extracts of plant, marine and microbial origin, and a novel class of *in vitro* active agents has been isolated from several cyanobacterial extracts. Collaborations with Dr. Paul Cox of Brigham Young University in the study of Samoan medicinal plants and Dr. Won Sick Woo of Seoul National University on Korean medicinal plants continue to be productive, and several of these medicinal plant extracts have exhibited some preliminary *in vitro* anti-HIV activity and are being investigated. A new collaboration with Kunming Botanical Research Institute in the study of Chinese medicinal plants has been established.

Presentations on the DTP natural products and screening programs have been made at a number of chemistry and pharmacognosy meetings, including an IUPAC symposium on natural products in Kyoto, an international coral reef symposium in Townsville, Australia, a seminar on plant antitumor agents in Champery, Switzerland, and the American Society of Pharmacognosy meeting in Park City, Utah.

ANNUAL REPORT OF THE BIOLOGICAL TESTING BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Biological Testing Branch (BTB) has responsibilities which include the development and implementation of a disease-oriented *in vitro* screening program for a large number of candidate cancer chemotherapy compounds and for follow-up *in vivo* testing of selected agents.

The BTB has responsibility for developing an AIDS testing program which includes the establishment and implementation of an anti-HIV screen (*in vitro*) with follow-up *in vivo* testing capabilities.

The BTB manages a large resource for the production, quality control, and distribution of genetically and biologically defined rodents. These disease free experimental animals are distributed to other NCI Divisions, intramural investigators at Bethesda and FCRF, other investigators within the NIH, other governmental agencies, and NIH grantee investigators on a cost reimbursement system.

The BTB maintains a large repository of experimental animal and human tumor lines for usage by DCT and other NCI programs. Tumors are also distributed to qualified cancer research investigators on a cost reimbursement basis.

The objectives of the BTB are to:

I. Cancer Area

1. Expand *in vitro* testing capabilities from the current capacity of approximately 50 human tumor lines to encompass approximately 100 well-characterized and defined lines of diversified origin representing the major types of cancer.

2. To expand *in vitro* screening capacity from the current level to our goal of 20,000 compounds on an annualized basis.

3. Develop models and enhance *in vivo* testing capacity to the extent that selected compounds from the *in vitro* screen can be tested for confirmation purposes. The time frame for accomplishing adequate *in vivo* testing capacity is dependent upon the renovation of space at FCRF (APA buildings).

II. AIDS Area

1. To implement *in vitro* screening capacity to an interim level of 10,000 compounds and a final level exceeding 40,000 compounds on an annualized basis.

2. To develop models and testing capacity to the extent that initial *in vivo* testing of candidate compounds from the *in vitro* screen can be accomplished. The time frame for reaching appropriate *in vivo* testing capacity is dependent upon the renovation of space at FCRF (APA buildings) and/or the utilization of extramural contracts.

III. Animal Production and Tumor Procurement

1. To continue to produce laboratory animals of highest quality (from both a health and genetic viewpoint) and necessary volume to meet the needs of the various programs using these services and to make the necessary adjustments to make this area as cost effective as possible.

2. To obtain a sufficient supply of human tumor cell line materials and xenografts, and to develop this material through extramural and FCRF efforts to the extent that a large number of well-characterized cell lines with a documented patient history are available for *in vitro* and *in vivo* testing.

Accomplishments:

I. Cancer Area

1. *In vitro* screen

During the past year, feasibility studies were accom- plished to assure the capability of testing 20,000 compounds annually in the *in vitro* screen against a large number of human tumor cell lines utilizing a selected protocol. Protocols are now developed and large scale testing will be implemented very soon.

2. Secondary testing

A. *In vitro*

Confirmatory protocols have been established for initial actives from the *in vitro* primary screen. Protocol selection will be based on tumor type and desired information.

B. *In vivo*

Model development studies have continued, but have been limited due to space and staffing problems. In the absence of quantitative data for potential evaluation models, it has been decided that the subcutaneous model which provides significant tumor inhibition/cell kill information, and which has been studied extensively in murine models will be utilized. *In vivo* testing will largely be accomplished extramurally until FCRF facilities are made available.

II. AIDS Area

1. *In vitro* screen

The *in vitro* screen has been implemented to the extent that testing has exceeded the 15,000 compound level on an annualized basis. The level of effort should be expanded for *in vitro* AIDS screening during the current fiscal year to exceed our goal of 40,000 compounds.

2. *In vivo* screen

Initial studies have been conducted with a microencapsulation model using the AIDS virus. Additional studies will be implemented as appropriate with bovine lentivirus models adapted to smaller animals. Additional feasibility studies will be conducted with the AIDS virus adapted to severely compromising immune deficient mice.

III. Animal Production and Tumor Distribution

1. Quality standards for animal production have been maintained. Animals have been supplied to qualified investigators which are free of pathogenic organisms and genetically sound. The payback system has continued to work well in making the animal production system more cost effective. Adjustments have been made in animal production to reflect changes in DTP objectives including more reliance on athymic mice and less overall volume of usage.

2. The tumor bank has expanded its capacity to accomodate a number of the cell lines utilized in the disease-oriented screening program. Steps have been taken to enhance the acquisition of cell lines from both the cancer and AIDS testing programs and to make these lines available for distribution. The payback system for cell line distribution is working successfully.

BIOLOGICAL TESTING BRANCH

FY 1989

PRIMARY RODENT CENTERS (3)	$3,201,461
Supply breeding nucleus for the animal program and athymic mice for drug evaluation.	
RODENT PRODUCTION CENTERS (1)	223,269
Large-scale production of nuce mice under barrier controlled environment.	
DIAGNOSTIC & HISTOCOMPATIBILITY PROJECTS (7)	430,182
To monitor animal health and genetic integrity.	
DEVELOPMENT OF STANDARDS & GUIDELINES (1)	40,000
For animal care and breeding.	
QUALITY CONTROL AND MODEL DEVELOPMENT (1)	966,258
PRIMARY SCREENING OF HUMAN AIDS VIRUS	576,670
FREDERICK CANCER RESEARCH FACILITY (2)	
Animal Production	$2,800,000 (EST.)
Minus Estimated Reimbursements	2,382,000
Estimated BTB Cost	$ 418,000
CANCER RESEARCH AND SUPPORT PROJECTS	$3,981,915 (EST.)
AIDS RESEARCH AND SUPPORT PROJECTS	$2,539,005 (EST.)
TOTAL ESTIMATED COST.	$12,376,760

Some of the above figures are ESTIMATES because the actual funding has not taken place as of this date.

ANNUAL REPORT OF THE INFORMATION TECHNOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Information Technology Branch (ITB) supports the data processing, management, and analysis needs for DTP's drug discovery and development programs in cancer and AIDS. ITB maintains large and detailed databases for the DTP. These record information on all chemical samples tested and virtually all biological test results obtained by the NCI anticancer screen since 1956. We maintain similar databases for the anti-AIDS screen initiated in mid-1987. The most consistent attribute of the DTP computing systems this year was that of change itself.

The key event of the year was the acquisition of a VAX 8820 facility at the NCI-FCRF complex at Frederick, Maryland. The new computer, now housed in its new Building 378, arrived in April, 1989. A team from Digital Equipment Corporation and the staff of the Advanced Scientific Computing Laboratory (ASCL) of PRI quickly put it in running order. The core of the system is the tightly-coupled two processor VAX 8820 cpu configured with 128 megabytes of memory. Two HSC 70s link the two cpu's to 14.4 Gbytes of magnetic disk storage. The entire complex links by Ethernet to the In Vitro Cancer and In Vitro AIDS laboratories and to the DTP offices in the Executive Plaza North Building in Bethesda.

This excellent facility is the heart of the DTP computing environment. It replaces the DEC-10 and IBM 370 systems previously available in a time-shared arrangement with DCRT. ITB planned for this arrival by instigating a variety of projects aimed at rewriting, redesigning, and generally modernizing various software systems. Fein-Marquart rewrote the chemistry system, a custom-designed hierarchial database, to accommodate the 32-bit architecture of the VAX. A specially chartered multi-contractor team redesigned the biological data files for cancer and AIDS. These formerly were ASCII flat files on the IBM 370. They were redesigned as a system of tables and loaded into Oracle 6.0 on the VAX. VSE took the ASSIGN/AVAIL program from an IBM environment and placed it in Oracle 6.0 (after a short period on a MicroVAX). The ITB computing system incorporates distributed processing in that microcomputers capture laboratory data and make end-result calculations. These link by Ethernet to the VAX. We upgraded the microcomputers from 286 microprocessor-based machines to 20MHz 386-based microcomputers. This processor change required changes in the custom multi-tasking software (IVSS).

Printing capabilities were considerably enhanced by the addition of two LPS40 laser printers. Unlike the existing large HP Laser Printer, these LPS40 printers have Postscript capabilities which will be heavily used by DTP application programmers. We were well along with the necessary conversions of existing AIDS and cancer graphic data displays by the time the new printers arrived. Of course, we must continue organization and coordination of the various conversion efforts if we are to complete successfully the transition from the DCRT computers to the dedicated VAX.

The transition from a very un-integrated, multi-site, poorly communicated and poorly coordinated system existing in a time-shared multi-mainframe environment to a well integrated, well communicated and well coordinated distributed processing system built around a dedicated advanced super minicomputer is the goal. The hardware is largely in place, and the main elements of software are equally at hand. The key now is to build in the required level of integration by appropriate interface strategies.

ITB management feels strongly that it must achieve the goal of an integrated computing environment. We feel that the key is in seeking solutions to the problems which inhibit full integration. In the current multi-contractor environment, this is done by chartering work groups comprised of the most appropriate people from several contracts. The design of the relational database tables from the ASCII flat files was an especially successful instance of a chartered work group. This group included the talents and efforts of several highly talented people from several contracts and from the ITB staff.

The changes described above were in hardware, software, and facilities. Equally significant this year were the changes made in the contractor support and in the way ITB management used that support to achieve the goals of DTP. We added several new contractors to the team: Capitol Technology and Information Services, Inc. (CTIS) gave fresh extra strength and indepth experience to the AIDS database design effort; Technical Resources Incorporated (TRI) provided excellent new insight, creativity, and technical expertise to the cancer database design team; and the truly outstanding staff and management of the ASCL contributed in a broad range of important capacities including a perfectly planned and executed acquisition of the VAX, a new building to house it, and the associated software and peripheral equipment. The ASCL group was also much involved in the database design team and did an outstanding job in the evaluation, selection, acquisition, and setup of Oracle 6.0. They also did an excellent job helping to resolve many difficulties in the Xenix-based IVSS microcomputer systems. In this context it seems important to note the continuing excellent contributions made by contractors who are not at all new to the DTP contractor support team:

Atlantic Research Corporation (ARC, formerly ORI) this year made significant contributions in diverse areas; for instance, their key input in the early design phases of the new VAX system, outstanding work in developing first-rate graphic display systems both for ad hoc interactive requests and for robust and efficient production systems capable of meeting DTP's stringent requirements for high quality, high volume graphic supplier report forms. ARC also played an outstanding lead role in the development of a rational set of relational database tables beginning with the old ASCII flat files. Also, they developed a suite of personal computer programs they call the Data Analysis System (DAS). The DAS may be, when completed, the most general and user friendly way for DTP scientific managers to access and analyze either AIDS or cancer data contained in Oracle on the VAX.

As mentioned above, the chemistry database system had to be rewritten for the 32-bit architecture of the VAX. This was a major contribution of significant importance to DTP. Not only was the basic chemistry system for synthetic compounds rewritten, it was also simultaneously upgraded and maintained as a functioning production system. This was done despite enormously difficult circumstances caused wholly by the accelerating decomposition of the DEC-10 (The DEC-10 served as an incompetent host over the entire life of the chemistry database). The excellent Fein-Marquart team managed to bring the entire conversion to a timely and highly successful conclusion. Simultaneously with the conversion project, they

developed two additional systems of major importance to DTP. One of these was a database system designed to control the vast new natural product acquisition and testing program. The other was a sophisticated inventory and sample management system for the pharmaceutical group within DTP.

Among the most notable contributions of the VSE group was the valuable new derivative program of DTP's microcomputer-based laboratory data collection and calculation program. They designed the new program and developed it in flawless form within two months time. It gives the AIDS laboratory staff a useful tool to manage the testing of large numbers of natural product fractions.

Two Phase II SBIR contracts continued their efforts to develop personal computer based versions of MLAB. Civilized Software Inc. plans to release this year its first version of a its MLAB designed to operate in a DOS environment. Creare Inc. is designing a version of MLAB it calls ML/e (spoken as "Emily"). ML/e will operate in a SCO Xenix environment.

Mr. Robert Brennan and Mrs. Marie Zehnacker, both of the ITB staff, programmed an extensive series of new data analysis programs. Dr. Phil Skehan devised the techniques involved in these analyses. The "C" language programming required to produce these complex analyses was difficult and tedious requiring several man-months to complete. The programs are now available in the In Vitro cancer laboratory for operation by the laboratory technical staff.

Mrs. Penny Svetlik of ITB was also highly productive this year. She collaborated with Dr. Dick Donovick to develop a cell line database system. She designed the program to collect, organize, and retrieve information collected in regard to the origins and characteristics of human tumor cell lines. Mrs. Svetlik also collaborated with Ms. Barbara Allen of TRI to coordinate classes in the VMS operating system which Ms. Allen taught to the staff of DTP. In addition, Mrs. Svetlik coordinated important projects of the branch including the redesign, rewriting, and rehosting to the VAX of application programs originally developed for the IBM 370. She was liaison between the ITB and the AIDS contract personnel at Southern Research Institute (SRI) in Birmingham, Alabama. This responsibility required her to travel to Birmingham on several different occasions. Later, she placed a special program in the SRI microcomputer so that she could operate the Birmingham computer from her office computer in Bethesda.

Other staff of ITB: Mrs. D. Upton, Mr. J. Waters, and Mr. A. Feldman all worked hard on diverse tasks. Some of these tasks were especially important to the branch, and many were important to people in other branchs of DTP.

ITB management wrote several programs of special interest this year. One of these tracks the progress of events from acquisition of samples through the screening process to generation and distribution of test results. The availability of this information together with the cooperative efforts of several DTP branches acted to minimize undue delays in distribution of conclusive screening results for the AIDS program. A similar program will be developed for the anticancer effort.

Techniques are available to express and display data from the anticancer screen as "fingerprint" patterns. These patterns can be shown to be similar for compounds related by structure or biochemical mechanism of action. We further refined the techniques and developed a

derivative application which provides a powerful tool to monitor the reproducibility of the patterns produced. ARC is revising these programs to run on the VAX.

Publications:

Marsh JC, Shoemaker RH, Salmon SE, Kern DH, and Venditti JM. Relationship between in vitro tumor stem cell assay and in vivo antitumor activity using the P388 leukemia, Int J Cell Cloning 1988;6:60-8.

Boyd MR, Shoemaker RH, Cragg GM, Suffness M. New avenues of investigation of marine biologicals in the anticancer drug discovery program of the National Cancer Institute. In: Jefford CW, Rinehart KL, Shield LS, eds. Pharmaceuticals and the sea. Lancaster: Technomic Publishing Co. Inc., 1988;27-44.

Shoemaker RH, McLemore TL, Abbott BJ, Fine DL, Gorelik E, Mayo JG, Fodstad O, Boyd MR. Human tumor xenograft models for use with an in vitro based, disease-oriented antitumor drug screening program. In: Winograd B, Peckham MG, Pinedo HM, eds. Human tumor xenografts in anticancer drug development. European School of Oncology Monograph. Berlin: Springer-Verlag, 1988;115-20.

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay, Cancer Res 1988;48:589-601.

McLemore TL, Eggleston JC, Shoemaker RH, Abbott BJ, Bohlman ME, Liu MC, Fine DL, Mayo JG, Boyd MR. Comparison of intrapulmonary percutaneous intrathoracic, and subcutaneous models for the propagation of human pulmonary and non-pulmonary cancer cell lines in nude mice, Cancer Res 1988;48:2880-6.

Kern DH, Shoemaker RH, Hildebrand-Zanki SU, Driscoll JS. Catechol analogs with selective cytotoxicity for human malignant melanoma: structure activity relationships, Cancer Res 1988;48:5178-82.

Vickers PJ, Dickson RB, Shoemaker RH, Cowan KH. A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth in vivo, Mol Endocrinology 1988;2:886-92.

Vince R, Hua M, Brownell J, Daluge S, Lee F, Shannon WM, Lavelle GC, Qualls KJ, Weislow OS, Kiser R, Cannonico PG, Schultz RJ, Narayanan VL, Mayo JG, Shoemaker RH, Boyd MR. Potent and selective activity of a new carbocyclic nucleoside (Carbovir: NSC 614846) against human immunodeficiency virus in vitro, Biochem Biophys Res Commun 1988;156:1046-53.

Shoemaker RH. An in vitro approach to disease-oriented antitumor drug screening: the human tumor colony forming assay. In: Davis BD, Ichikawa T, Maeda K, Mitscher LA. eds. Horizons on antibiotic research. Tokyo: Japan Antibiotics Research Association, 1988;66-73.

Boyd MR, Shoemaker RH, McLemore TL, Johnston MR, Alley MC, Scudiero DA, Monks A, Fine DL, Mayo JG, Chabner BA. Drug development. In: Roth JA, Ruckdeschel JC, Weisenburger TH, eds. Thoracic oncology. Philadelphia: WB Saunders Co. 1989;711-22.

Weislow OS, Kiser R, Fine DL, Bader J, Shoemaker RH, Boyd MR. New soluble- formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for aids-antiviral activity, JNCI 1989;81:1577-86.

Paull KD, Shoemaker RH, Boyd MR, Parsons JL, Risbood PA, Barbera WA, Sharma MN, Baker D, Hand E, Scudiero D, Monks A, Alley M, Grote M. The synthesis of XTT: a new tetrazolium reagent bioreducible to a water soluble formazan, J Hetero Chem 1988;25:911-14.

Plowman J, Paull KD, Atassi G, Harrison SD Jr, Dykes DJ, Kabbe HJ, Narayanan VL, Yoder OC. Preclinical antitumor activity of batracyclin (NSC 320846), Invest New Drugs 1988;6:147-53.

Plowman J, Harrison S, Dykes D, Paull K, Narayanan VL, Tobol HK, Martin J, GriswoldDP Jr. Preclinical antitumor activity of an alpha-picoline derivative, penclomedine(NSC 33720), Cancer Res 1988; 49:1909-15.

Scudiero D, Shoemaker R, Paull K, Alley M. Monks A, Tierney S, Nofzinger T., Currens, M., Burkew D, Boyd M. Evaluation of a soluble tetrazolium/formazan assay for growth and drug sensitivity in culture, Cancer Res 1988;48:4827-4833.

Shoemaker RH, Monks A, Alley MC, Scudiero DA, Fine DL, McLemore TL, Abbott BJ, Paull KD, Mayo JG, Boyd MR. Development of human tumor cell line panels for use in disease-oriented drug screening, Prog Clin Bio Res 1988;276:265-86.

Nasr M, Paull KD, Narayanan VL. Computer assisted structure-anticancer activity correlations of organotin compounds. In: Zuckerman JJ, ed. Tin and malignant cell growth. CRC Press, 1988;211-26.

Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, Boyd MR. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and compare algorithm, JNCI (In press).

ANNUAL REPORT OF THE PHARMACEUTICAL RESOURCES BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Pharmaceutical Resources Branch (PRB) is structured to provide comprehensive pharmaceutical services to the various Programs of the Division of Cancer Treatment and recently, to provide similar services to the AIDS Clinical Programs of the National Institute of Allergy and Infectious Disease (NIAID). The primary objectives of the Branch are to supply high quality chemical substances and formulated products for investigative program use. These objectives are accomplished essentially through contract support activities. During this report period, the Branch supervised a total of 23 contracts with a combined annual budget of approximately seven million dollars.

The major contract areas include: chemical preparations and pilot plant production, analytical services, pharmaceutical research and development, and pharmaceutical manufacturing. Additionally, the Branch is responsible for shelf life surveillance, storage and distribution, and computerized inventory maintenance of all drug products used in the Clinical Programs of the Division of Cancer Treatment (DCT).

Staff

The Pharmaceutical Resources Branch is presently staffed with eight senior professionals, one technical and two secretarial personnel. The classification of the senior professionals is as follows: five PHS Commissioned Corps Pharmacists, one Ph.D. analytical chemist, and two Ph.D. medicinal chemists.

The Branch consists of four functional areas:

Chemical Resources

The primary functions of Chemical Resources are to provide for resynthesis, large-scale production and procurement of chemical substances. These services are accomplished by the management and supervision of a contract program for resynthesis and pilot plant production of various quantities of bulk substances primarily intended for pharmaceutical manufacture of investigational products for clinical evaluation. Chemical Resources supervises eight chemical prep lab contracts consisting of a combined annual effort of approximately 34 man years.

An important aspect of Chemical Resources is the interaction with chemical suppliers of bulk drug substances. The PRB chemists must contact the suppliers and ascertain that materials they are to provide are prepared under FDA required Good Manufacturing Practices (GMP) and are of the highest quality possible. An increasing quantity of chemical bulk substances are being provided by suppliers for PRB contract formulation.

Analytical

The analytical service provides for: (1) analytical characterization of new investigational agents and (2) analytical assessment of chemical and formulated products. The analytical service supervises contractors engaged in the development of analytical methodology to determine the purity of chemicals, potency of active ingredients in pharmaceutical formulations, stability of formulated products under accelerated and simulated use conditions, and identification of impurities and/or degradation products. The analytical chemist prepares bulk chemical specifications used for acceptance criteria of additional supplies either from commercial sources or chemical preparation contractors. The specifications and validated analytical methodology are prepared in a format suitable for submission to the Food and Drug Administration as part of the NCI's Investigational New Drug Application. The analyst is also responsible for chemical evaluation of new investigational dosage forms.

Presently, the analytical chemist supervises four analytical contracts representing a combined 18 staff year effort. These contractors have the expertise to chemically characterize a very structurally diverse group of chemicals. These contractors are also responsible for the development and application of stability-indicating methods for all new drug substances.

Analytical data developed on new investigational compounds is assembled and published in a book entitled "NCI Investigational Drugs - Chemical Information". This text contains stability-indicating methods, spectral data, approximate solubility and stability data, and other appropriate information on a large number of agents. The publication is distributed on request and without charge to investigators throughout the country.

Pharmaceutical Research and Development

The dosage form development component is responsible for conversion of bulk chemicals into pharmaceutical products suitable for clinical use in chemotherapy and AIDS programs. About one-half of the drugs required for intravenous delivery do not exhibit adequate solubility or stability and some form of pharmaceutical intervention is required. Standard approaches (salts, solvents and surfactants) are initially evaluated. Emphasis is also given to evaluation of newer techniques to improve solubility or stability (emulsions, prodrugs and complexation). The developed dosage form is evaluated for chemical content, antitumor activity in the rodent screen, and feasibility for manufacture on production scale.

All of the production development effort is conducted under contract with the Pharmaceutical Development staff servicing as project monitors.

The Product Development service is responsible for the supervision and management of two pharmaceutical R & D contracts with a combined annual effort of eight man years, one combined R & D (one and one-half man years), and a pharmaceutical contract.

Pharmaceutical Acquisition and Production

The Pharmaceutical Acquisition and Production manages six pharmaceutical contracts with capabilities to produce a broad variety of pharmaceutical products. The service manages a storage and distribution contract with computer capabilities for accurate accountability of the disposition of all investigational products, and also manages a shelf life contract involving an annual three and one-half man year effort.

In addition, the service manages a sizeable intramural budget for the direct purchase of chemicals and formulated products. During this report period, drug purchase expenditures were in excess of 2.5 million dollars. A similar effort was begun during the year to record the expenditures for drugs for use in the AIDS program. This involved establishing a dual record system to accurately account for the expenditures for AIDS drugs.

A significant amount of staff time is expended in preparing purchase specifications, award justifications, and performing financial recordkeeping functions. Several different NIH mechanisms to obtain contracts are utilized to obtain drugs, such as blanket purchase agreements, indefinite delivery contracts, direct purchase contracts, etc.

The contractors managed by the Pharmaceutical Acquisition and Production produced over 700,000 injectable units, and slightly less than 400,000 oral dosage forms for clinical distribution.

Investigational product literature in the form of Investigational Drug - Pharmaceutical Data Sheets is prepared by the staff. These information sheets are also supplied in bound book form (NIH Publication No. 86-2141) which is updated periodically. During this reporting period, over 4,300 issues were distributed.

Goals and Accomplishments

It is noteworthy that the branch accomplished the primary extraction of bugula neritima for purification and preparation of bryostatin. Also, the isolation of more than 400 Gm of taxol was accomplished during this reporting period.

The branch was responsible for manufacturing large quantities of bulk and formulated supplies of dideoxyinosine for adult and pediatric Phase I trials. This involved a major effort of several prep lab and pharmaceutical contractors and staff for quick response to rapidly increasing demands of drug while awaiting supplies from the clinical sponsor.

We anticipate four to six new agents from the AIDS HIV screen to be approved for development during the next reporting period. In addition, we anticipate another two to three candidate agents provided from non government sources that may require product development for AIDS trials. A

contract program is in place and all new agents will receive a high priority for development.

During this reporting period the branch experienced a significant increase in the receipt and distribution of a variety of biological products. These products usually require dry ice or wet ice packaging and special mailing procedures. These procedures are labor intensive and expensive and have resulted in administrative and financial contract modifications.

During the next reporting period the PRB will concentrate on evaluating new parenteral drug delivery systems such as with liposomes, emulsions and micro particles for intravenous use.

Also, during this reporting period a computerized Pharmaceutical Data System was implemented. This system is designed by an SBIR contractor to track analytical, chemical, pharmaceutical, and distribution actions of DCT investigational agents. The system is expected to improve drug product files and make more efficient use of staff time.

Publications by Staff

1. Flora KP, Quinn FR, Milne GWA, Damiano VA, Trissel LA, Cradock JC, Davignon JP, Kleinman LM, Morgan JM, Vishnuvajjala BR, Koss DA Jr, Murrill E, West N, Ray G, Crook MV, Schierhoff A, Skinner T. NCI investigational drugs: chemical information 1988. Bethesda, MD: National Cancer Institute, 1988; DHHS publication no. (PHS)88-2654.

2. Trissel LA, Davignon JP, Flora KP, Quinn FR, Vishnuvajjala BR. NCI investigational drugs: pharmaceutical data 1988. Bethesda, MD: National Cancer Institute, 1988; DHHS publication no. (PHS)88-2141.

3. Flora KP. Software review: MINSQ 2.3, Am J Hosp Pharm, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07183-03 PRB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The influence of molecular structure on chemical and biological properties

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI : Frank R. Quinn Chemist PRB, NCI

COOPERATING UNITS *(if any)*

James V. Silverton, LC, NHLBI
Rudiger D. Haugwitz, DSCB, NCI

LAB/BRANCH

Pharmaceutical Resources Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

X-ray crystallographic, energy minimization and quantum mechanical calculations are being employed on compounds of biological interest to give insights into and explanation of their modes of behavior.

Various compounds showing promise against the AIDS virus are being systematically investigated to obtain structural and electronic properties which may help elucidate the mechanism of their action and thus lead to improved analogs. The x-ray structures of 2',3'-dideoxyinosine (DDI) and 2',3'-dideoxyguanosine are being determined. Strain energies and quantum calculations are being carried out on these compounds.

PHS 6040 (Rev 1/84) GPO 914-918

ANNUAL REPORT OF THE PHARMACOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Pharmacology Branch (PB) is involved with two basic aspects in the preclinical drug developmental program: detailed therapeutic studies and pharmacokinetic studies on candidate agents for cancer and AIDS treatment. Via the contract mechanism, special studies are technically managed to explore the in vivo therapeutic efficacy of new drugs in murine models with various routes of administration and treatment schedules. For cancer drugs, advanced stage tumors, metastatic disease and drug resistant tumors are of prime interest. For AIDS drugs, murine retroviruses (Rauscher and LP-BM5) are the current prime models but the SCID and transgenic mouse are being considered. Separate contracts are used to obtain methods of analysis for antitumor and anti-AIDS agents of interest to our program and to conduct detailed pharmacokinetic studies in order to better understand their in vivo behavior.

Numerous drug candidates were tested for cancer in detailed therapeutic studies: CPE-C (NSC 375575), Carmethizole (NSC 602668), Pyrazoloacridine (NSC 366140), Penclomedine (NSC 338720), 8-Cl-cAMP (NSC 614491), Rapamycin prodrug (NSC 606698D) and three morpholino-anthracyclines (NSC 354646, NSC 357704 and NSC 619003D). The latter three anthracycline analogues were of particular interest because of indications in vitro for effectiveness against multidrug resistant (Mdr) tumors. In vivo tests against the Mdr P388/Adr and the 16/C mammary adenocarcinoma have not yet demonstrated this advantage over adriamycin although their potency is much greater than that of adriamycin.

In detailed therapeutic studies for AIDS two activities were ongoing. A contract was put into operation to identify agents with activity against the dihydrofolate reductase from Pneumocystis carinii and was later smoothly transfered to NIAID.
Initial agents identified by the anti-HIV FCRF screen are undergoing evaluation in murine retrovirus models both in vitro and in vivo. Background data were obtained for the in vivo activity of AZT against the Rauscher MuLV.

During this year three new AIDS pharmacology contracts and three renewed cancer pharmacology contracts were put into operation. Five novel compounds with demonstrated in vitro anti-HIV activity are currently being studied. The phosphorothioate oligonucleotides, poly-C 28-mer (NSC 613671), antisense to HIV rev gene 28-mer (NSC 613672) and a discreet analog (NSC 624958D) are undergoing method development, as is a discreet synthetic compound (NSC 615985D). Identification of the metabolites of the HIV active compound Carbovir (NSC 614846) continues. Five anticancer agents have

undergone complete method development and detailed pharmacokinetic studies: Penclomedine, Batracylin, Histidinol, 8-Cl-cAMP, Cyclopentenyl cytosine. Another three, Cyanomorpholinoadriamycin, O6-Methylguanine and the prodrug of Rapamycin (NSC 606698D) are at various stages of method development and pharmacokinetic analysis.

PUBLICATIONS BY STAFF

Clagett-Carr K, Sarosy G, Plowman J, Hoth DF, Leyland-Jones B. NMF: Cytotoxic, radiosensitizer or chemosensitizer. J Clin Onc 1988;6:906-18.

Hornung HL, Back TC, Zaharko DS, Urba WJ, Longo DL, Wiltrout RH. Augmentation of natural killer activity, induction of IFN and development tumor immunity during the successful treatment of established murine renal cancer using flavone acetic acid and IL-2. J Immunol 1988;141:3671-79.

Marquez VE, Lim MI, Treanor SP, Plowman J, Priest MA, Markovac A, Khan MS, Kaskar B, Driscoll JS. Cyclopentenylcytosine. A carbocyclic nucleoside with antitumor and antiviral properties. J Med Chem 1988;31:1687-94.

O'Dwyer PJ, King SA, Plowman J, Grieshaber CK, Hoth DF, Leyland-Jones B. Pyrazole, Role in chemotherapy? Invest New Drugs 1988;6:305-10.

Plowman J, Paull KD, Atassi G, Harrison SD Jr, Dykes DJ, Kabbe HJ, Narayanan VL, Yoder OC. Preclinical antitumor activity of Batracylin (NSC 320846). Invest New Drugs 1988;6:147-153.

Plowman J, Harrison SD Jr, Dykes DJ, Paull KD, Narayanan VL, Tobol HK, Martin J, Griswold DP Jr. Preclinical antitumor activity of an alpha-picoline derivative, penclomedine (NSC 338720), on human and murine tumors. Cancer Res 1989;49:1909-15.

Paull KD, Shoemaker RS, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, Boyd MR. Display and analysis of patterns of differential activity of drugs against human tumor cell lines:development of mean graph and compare algorithm. JNCI 1989;81: (in press).

PUBLICATIONS BY CONTRACTORS:

Brodfuehrer JI, Moore DJ, Melder DC, Wilke TJ, Powis G. In vitro cytotoxicity of pyrazine-2-diazohydroxide: specificity for hypoxic cells and effects of the microsomal coincubation. Invest New Drugs 1988;6:3-9.

Brodfuehrer JI, Powis, G. A gas chromatographic assay for the new antitumor agent sulfamic acid diester (NSC 329680) and its stability in buffer, and plasma. J Chromatog Biomed Appl 1988;427:247-55.

Brodfuehrer JI, Wilke TJ, Powis G. Pharmacokinetics and metabolism of the antitumor agent sulfamic acid, 1,7-heptanediyl ester (sulfamic acid diester) in the mouse and beagle dog. Cancer Chemother Pharmacol 1988;22:120-25.

Frank SK, Mathiesen DA, Kuffel MJ, Szurszewski M, Ames MM. Preclinical pharmacology of the anthrapyrazole analog oxantrazole (NSC 349174). Cancer Chemother Pharmacol 1989;23:213-18.

Kalin JR and Hill DL. Determination of 2′,3′-dideoxyadenosine, 2′,3′-dideoxyinosine and 2′3′-dideoxycytidine in biological samples. J Chromatog Biomed Appl 1988;431:419-23.

Moore DJ, Brodfuehrer JI, Wilke TJ, Powis G. Disposition and Metabolism of the antitumor agent pyrazine-2-diazohydroxide in mouse and beagle dog. Cancer Chemother Pharmacol 1988;21:269-73.

Rose LM, Tillery KF, El Dareer SM and Hill DL. High-performance liquid chromatographic determination of doxorubicin and its metabolites in plasma and tissue. J Chromatog Biomed Appl 1988;425:419-23.

Waud WR and Hill DL. High-performance liquid chromatographic determination of terephthalamidine in plasma. J Chromatog Biomed Appl 1988;425:220-26.

Ames MM, Mathiesen DA. High-performance liquid chromatographic assay and preclinical pharmacologic studies with the experimental antitumor agent batracylin. J Chromatog Biomed Appl 1989 (in press).

Brodfuehrer JI, Wilke TJ, Kinder DH, Powis G. Preclinical pharmacology studies in mouse and beagle dog with the new antitumor agent carmethizole (NSC 602668). Cancer Chemother Pharmacol 1989 (in press).

El Dareer SM, Tillery KF, Kalin JR and Hill DL. Disposition of 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine in mice. Invest New Drugs 1989 (in press).

ANNUAL REPORT OF THE TOXICOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The objectives of the Developmental Therapeutics Program center on the discovery and preclinical development of agents with anticancer, and presently, with anti-HIV potential. Studies focusing on the hazards of new investigational agents to healthy organs in intact experimental animals are the final steps in the preclinical stages of new drug development. These investigations comprise the primary responsibility of the Toxicology Branch and are carried out by studies in two stages, IIA and IIB, corresponding to the preclinical steps in the DCT Decision Network process. In stage IIA, the maximally tolerated dose of the drug (MTD) is determined in rodents and dogs on several administration schedules and the kinetics of elimination are established in dogs. In stage IIB, full scale Investigational New Drug Application (INDA)-directed toxicology studies are performed using the MTD from the earlier stage as the pivotal dose. Scheduling is established from the preclinical efficacy and pharmacology studies, the stage IIA toxicology studies and the projected clinical regimen. These stage IIB studies have the following objectives:

1. Determine end-organ toxicities and additionally establish the dose-responsiveness and schedule-dependency of toxicity;

2. Determine plasma drug concentrations and correlate levels to safety, toxicity and <u>in vitro</u> efficacy;

3. Determine the reversibility of toxicity during a post-dosing recovery period;

4. Establish a safe starting dose for the Phase I clinical trials in humans.

This year nine new antineoplastic drugs or analogs and two anti-HIV agents were in the IIA phase of preclinical toxicology. Two drugs, CPE-C and pyrazoloacridine moved on to stage IIB. One drug, carmethizole, was dropped due to cardiotoxicity.

<u>Drugs in DN Stage IIA on which preliminary toxicity studies were performed</u>

Anticancer

Cyclopentenylcytosine	375575[a]
Pyrazoloacridine	366140[a]

Penclomidine	338720
Carmethizole	602668[b]
8-chloro cyclic AMP	614491
Stannous Purpurin	619679
O^6-Methylguanine	037364
Discreet	615291D
Discreet	606698D

Anti-HIV

Carbovir	614846
Discreet	615985D

[a]Moved to Stage IIB
[b]Dropped

Drugs in DN Stage IIB on which full-scale toxicity studies were performed

Rapamycin	226080
Batracylin	320846
Cyclodisone	348948
Tetraplatin	363812

Two drugs completed the IIB stage in the past year, Batracylin and L-BSO/L-PAM.

The information generated from the toxicology and pharmacology studies, particularly those carried out under Stage IIB, comprise the major portion of the evaluable preclinical information required by the Food and Drug Administration for an Investigational New Drug Application (INDA). The Division of Cancer Treatment maintains a master file with the FDA which contains toxicity study protocols for potential anticancer drugs. These protocols set forth jointly agreed to procedures for animal toxicity studies of antineoplastic drugs. Data from studies conducted under the protocols are accepted for regulatory purposes in INDA approval. The Toxicology Branch has continually amended the protocol files to accurately reflect newer methods and techniques. Individualized protocols (drug specific) are routinely developed from these basic designs to account for agent specific chemical and/or physical properties. Two reviewing divisions of the Center for Drugs, Oncology and Anti-Viral Drug Products as well as the Center for Biologics refer to this master file.

During FY 1989, the Branch continued two innovative projects designed to provide more definitive information on human toxicity of investigational agents. These projects include studies on in vitro toxicity of new drugs in bone marrow committed bone marrow stem cell compartments yielding a comparative hazard profile across species lines. Seven new anti-HIV drugs and four antineoplastic agents were comparatively

tested on murine and human committed bone marrow stem cells. In almost all cases, murine cells were more "sensitive" to the drugs than human cells confirming the safety of rodent toxicity data. The second project is comparative metabolism of anti-HIV agents in lymphocytes from experimental species and humans. In the case of nucleoside analogs for the treatment of HIV infections, the agent must be phosphorylated to its triphosphate in order to be incorporated into the growing oligodeoxynucleotide chain during reverse transcriptase directed DNA synthesis. Currently, carbovir metabolism is under comparison to ddI in human lymphocytes, both freshly isolated and in immortalized cell lines.

These preclinical studies enable the program to evaluate animal toxicity and potential human toxicity of compounds in early development, as well as to evaluate the pharmacokinetic and metabolic characteristics prior to committing the program to full scale drug development.

Publications:

Manuscripts:

O'Dwyer, PJ, King SA, Plowman J, Grieshaber CK, Hoth DF, Leyland-Jones B. Pyrazole: preclinical reassessment, Invest New Drugs 1988;6:305-10.

Christian MC, Wittes RE, Leyland-Jones B, McLemore TL, Smith AC, Grieshaber CK, Chabner BA, Boyd MR. 4-Ipomenaol: a novel new drug for lung cancer, JNCI, In Press.

Smith AC, Liao JTF, Page JG, Wientjes MG, Grieshaber CK. Pharmacokinetics of buthionine sulfoximine (BSO, NSC-326231) and its effect on melphalan induced toxicity in CD2F1 mice, Cancer Res, In Press.

Book Chapter:

Smith AC. The pulmonary toxicity of nitrosoureas. In: Gram TE, ed. Pharmacology and therapeutics. Great Britain: Pergamon Press, 1989;41(3)443-60.

ANNUAL REPORT OF THE GRANTS AND CONTRACTS OPERATIONS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

This Branch provides an administrative and managerial focal point for DTP extramural activities. These activities encompass all grants, contracts and cooperative agreements such as those involving the National Cooperative Drug Discovery Groups (NCDDGs). Activities for this year are summarized below by funding mechanism.

Grants

A breakdown of the grants in the Biochemistry and Pharmacology Program by topic subcategory is shown in Table 1. At the end of FY 1988, 310 grants totaling $49,239,000 were administered by the Branch. During FY 1989 a total of 384 grant applications were received. About 25% are expected to be awarded by the end of the fiscal year. These investigator-initiated projects comprised all aspects of drug discovery and development research. During the past year the referral guidelines for the program were updated to reflect current research priorities and to incorporate recent advances in modern drug design and development, such as computer modeling and molecular biological techniques. Research priorities include the discovery of new drugs and treatment strategies, selective targeting of therapeutic agents, development of new preclinical models for drug discovery, and understanding, preventing and overcoming drug resistance.

Contracts

The Branch coordinates all phases of the DTP contract process. Activities include coordinating the preparation of concept paragraphs for new and recompeting projects, overseeing the drafting of the technical portions of the Requests for Proposals (RFPs), arranging and monitoring the Source Evaluation Group (SEG) process, and editing the technical portions of the source selection documents. RFPs were issued and contracts awarded during the year for the support of both cancer and AIDS drug discovery and development projects. The title of each solicitation and the associated three to five year costs are listed in Table 2. In all, the Program awarded twenty-four contracts at a total value of $51,938,141 over the next three to five years. RFPs representing $34,914,181 in additional three to five year funding (pending award) were issued during the year.

The Branch maintains complete master files and monitors the Program reporting and technical merit review requirements for all active projects over the three to five year life of the contract. The number of ongoing contracts now exceeds one hundred.

Cooperative Agreements

The NCDDG Program was initiated in 1983 to exploit exciting developments in biomedical research for the discovery of new and more effective treatments for

cancer. Multidisciplinary and multi-institutional teams of the nation's most talented scientists from academic, non-profit research and commercial organizations are brought together to conceive and develop new drug and treatment strategies and novel models which will more accurately predict the efficacy of proposed treatments. NCI serves as a partner with each Group to facilitate research efforts. Currently there are nine funded Groups involving 43 laboratories in 33 institutions at an annual cost of $4,882,000 (Table 3). During the past year the program was expanded by the establishment of National Cooperative Natural Products Drug Discovery Groups, an effort to stimulate the discovery of new agents from natural sources, such as from tropical rain forests and marine habitats. Awards are planned by the end of September for the most meritorious applications. In an effort to stimulate the NCDDG approach to drug discovery still further, RFAs (Requests for Applications) were released this year to solicit new applications in three areas: general mechanism of action, a disease-oriented strategy, and novel model development. Awards are expected in early 1990.

The NCDDG Program has been successful in bringing new therapies to clinical trial. During the past year four new agents have entered Phase I clinical testing as a result of findings on NCDDG projects. Hycamptamine, an analog of camptothecin with improved water solubility which shows activity against several murine leukemic and solid tumor models and is a potent inhibitor of the interaction of topoisomerase I and DNA, entered clinical trial in March 1989. Collaborative clinical trials with NCI are expected to start in the near future. HomoDES, a potent inhibitor of polyamine biosynthesis, entered limited Phase I clinical trials at the University of Florida, Gainesville, FL. Another Group is examining toxicity and tumor localization of ^{111}Indium-labeled monoclonal antibody 225 IgG1 which binds with high affinity to the human EGF receptor and blocks EGF-induced activation of tyrosine kinase. Initial trials are being limited to patients with squamous cell lung carcinoma, a tumor which expresses increased numbers of EGF receptors. Finally, a novel diphtheria toxin-related interleukin-2 fusion protein was produced and introduced into clinical testing during 1989. Patients with T-cell leukemias or lymphomas which bear high affinity interleukin-2 receptors will be candidates for this new therapy.

Workshops

Last year the Branch cooperated with the Biological Response Modifiers Program (BRMP) in sponsoring a one-day workshop entitled "Specific Cancer Cell Targeting Using Molecular Genetic Technology." Based on new developments in this emerging area of research, the Branch recently issued a Program Announcement on this topic, with the BRMP serving as a co-sponsor. An example of the type of research being encouraged is the use of tissue specific promoters and enhancers to regulate the selective expression of inserted genes encoding cytotoxic molecules. The Branch also assisted in planning the conference on "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression: Therapeutic Implications" which was held on June 18-21, 1989. In addition to providing a forum for international experts to discuss recent developments in this relatively new research area, the conference assisted staff in formulating plans to foster the development of new therapies for cancer, AIDS and viral diseases based on antisense inhibitors.

Publications

1. Grem JL, Cheson BD, King SA, Leyland-Jones B, Suffness M. Cephalotaxine esters: Antileukemia advance or therapeutic failure? J Natl Cancer Inst 1988;80:1095-1103.

2. Marsh JC, Shoemaker RH, Salmon SE, Kern DH, Venditti JM. Relationship between in vitro tumor stem cell assay and in vivo antitumor activity using the P388 leukemia. Int J Cell Cloning 1988;6:60-8.

3. Suffness M, Abbott B, Statz D, Wonilowicz E, Spjut R. The utility of B16 melanoma and colon carcinoma 38 for in vivo screening or plant extracts. Phytotherapy Res 1988;2:89-97.

4. Suffness M, Newman DJ, Snader K. Discovery and development of antineoplastic agents from natural sources. In: Scheuer P, ed. Bio-organic marine chemistry. Heidelberg: Springer-Verlag, 1989, in press.

5. Suffness M, Thompson JE. National Cancer Institute's role in the discovery of new antineoplastic agents. In: Fautin DG, ed. Biomedical importance of marine organisms. San Francisco: California Academy of Sciences, 1988; 151-57.

TABLE 1

BIOCHEMISTRY AND PHARMACOLOGY GRANTS

PROGRAM AWARDS BY SUB-CATEGORY

FY 1988 (ESTIMATED)

A	Synthesis and Chemistry	81	$12,531,026
B	Natural Products	29	$ 3,051,901
C	Screening and Experimental Therapeutics	16	$ 3,542,127
D	Comparative Pharmacology	29	$ 3,150,370
E	Other Preclinical Aspects	3	$ 353,230
F	Mechanism of Action	114	$17,215,119
	Program Projects	5	$ 5,391,918
	Total	310	$49,239,000

Includes Traditional (R01), New Investigator (R23/R29), Small Business Innovation Research (R43/R44), Academic Research Enhancement (R15), Merit (R37) and Outstanding Investigator (R35) Awards. Does not include Conference (R13) or Equipment (S15) Awards.

TABLE 2

TOTAL CONTRACT VALUE (ISSUED/AWARDED)

ANTITUMOR PROJECTS

Preclinical Pharmacology Investigations of Antitumor Agents	1,621,155
Quality Control and Model Development in Rodents	5,495,944
Master Agreements for Large Scale Isolation of Antitumor Agents from Natural Sources	2,762,815
Preparation of Radiolabeled Materials	1,931,018
Literature Surveillance and Selections of Promising Natural Products	140,555
Procurement of Human Breast Cancer Cell Lines	499,355
Procurement of Prostate Cancer Cell Lines	945,750
Master Agreements for Chemical Synthesis	1,418,625
Primary Rodent Production Centers	11,024,880
Development of Dosage Forms and Delivery Systems for New Antitumor Agents	1,134,900
Development and Production of Pharmaceutical Dosage Forms	1,576,250
Partial Support of Institute of Laboratory Animal Resources	237,500
Detailed Drug Evaluation and Development of Treatment Strategies for Chemotherapeutic Agents	4,144,224
Collection of Shallow Water Marine Organisms	1,090,000
Total	$34,022,971

ANTI-AIDS PROJECTS

DTP AIDS Screening Data Base Support	1,043,492
AIDS DTP Computer DIS Installation	278,816
Mechanistically-oriented Anti-HIV Drug Prescreens	18,705,257
Development and Manufacture of Oral Dosage Forms of Anti-AIDS Agents	3,315,379
Preclinical Toxicology and Pharmacology of Drugs Developed for AIDS and Related Illnesses	5,784,418
Dosage Form Development of New Agents for the Treatment of AIDS	430,598
Large Scale Preparation of Anti-AIDS Drugs for Preclinical Toxicology and Phase I Clinical Studies	931,303
Large Scale Preparation of Anti-AIDS Drugs for Preclinical Toxicology and Phase I Clinical Studies (By Small Business)	1,044,191
Large Scale Preparation of Anti-AIDS Drugs for Phase II and III Clinical Trials	3,310,120
Large Scale Preparation of Anti-AIDS Drugs for Phase II and III Clinical Trials (By Small Business)	1,655,863
Total	$36,499,437

TABLE 2 (Continued)

ANTITUMOR AND ANTI-AIDS PROJECTS

Selective Acquisition of Compounds for Anticancer and Anti-AIDS Screening	4,185,715
Cultivation of Marine Anaerobic Bacteria	384,776
Cultivation of Marine Protozoa	784,723
Operation and Maintenance of DTP Biological Data Processing System	5,304,606
Computer Support Task Orders	1,823,458
Storage and Distribution of Chemicals and Drugs Used in Preclinical Evaluation and Development	3,846,636
Total	$16,329,914
GRAND TOTAL	$86,852,322

TABLE 3

NATIONAL COOPERATIVE DRUG DISCOVERY GROUP PROGRAM

TOTAL COSTS FOR FY 1989

Grant Number	Investigator/Institute/Title	Costs
5 U01 CA 45967-03	Brattain, Michael G. Baylor College of Medicine Growth Regulation of Human Colonic Neoplasms	$ 423,485
5 U01 CA 45962-03	Corbett, Thomas H. Wayne State University Drug Discovery - Anticancer Agents for Colorectal Cancer	$ 553,573
5 U01 CA 46088-03	Johnston, Michael R. University of Colorado Health Science Center Targeted Therapy for Lung Cancer	$ 641,393
5 U01 CA 37655-05	Levin, Victor A. M.D. Anderson Cancer Center Approaches to the Inhibition of Oncogene Expression	$ 412,005*
5 U01 CA 37641-05	Mendelsohn, John Memorial Sloan Kettering Cancer Center Anti-Receptor Monoclonal Antibodies in Cancer Treatment	$ 707,733
5 U01 CA 48626-02	Murphy, John R. The University Hosptial, Boston Growth Factor Receptor Targeted Toxins for Leukemia/Lymphoma	$ 603,626
5 U01 CA 37606-05	Porter, Carl W. Roswell Park Memorial Institute Inhibitors of Polyamine Biosynthesis and/or Function	$ 719,825*
5 U01 CA 40881-06	Ross, Warren E. University of Louisville Topoisomerases as New Therapeutic Targets	$ 601,169

TABLE 3 (Continued)

5 U01 CA 48105-02	Wahl, Geoffrey M. The Salk Institute Detection and Curing of Amplified Genes in Human Cancer	$ 219,191
	Total	$4,882,000

#FY 1988 Figures

ANNUAL REPORT OF THE LABORATORY OF BIOLOGICAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Laboratory of Biological Chemistry (LBC) is responsible for identifying as targets for drug design, cellular reactions that are critical to the control of tumor cell proliferation or differentiation. Recent advances in cell biology are evaluated for possible targets. Agents are designed to interact with these targets and are evaluated for biochemical and antitumor effectiveness. An important aspect of this mission is to develop appropriate in vivo systems to evaluate the chemotherapeutic effectiveness of agents shown to be active in simpler in vitro model systems. Accordingly, the LBC is involved in identifying endogenous factors present in vivo that modify drug action and influence differential toxicity with the aim of manipulating these factors to enhance antitumor activity. Approximately 75% of the LBC's resources is applied to non-traditional targets for antitumor drug design and study. These non-traditional targets include early key biochemical events signaling cell proliferation or differentiation. The other 25% of LBC resources is applied to the study of either traditional targets or active compounds with traditional or unknown mechanisms of action.

Reduced requirements for stimulation by growth factors may be the fundamental characteristic of transformed (neoplastic) cells. Research in molecular biology has identified several specific biochemical changes produced by introduction or over expression of oncogenes which may reduce the levels of exogenous growth factors needed to trigger cell replication. These findings suggest that a more selective approach to chemotherapy may focus on the interaction of growth factors with cells rather than on basic metabolic reactions such as those involved in nucleic acid synthesis. We have therefore initiated projects to develop new chemotherapeutic agents to block the action of growth factors. Non-traditional targets selected for drug design and study include: second messengers inositol triphosphate and diacylglycerol; myristoylation of cellular oncogene products; protein kinase C; and selected G-proteins.

The products of two distinct oncogenes (src and ros) are associated with increased levels of phosphatidylinositol polyphosphates in the membranes of cells transformed by these oncogenes. Test systems have been developed to identify drugs that inhibit phosphatidylinositol turnover or synthesis. A series of analogs of myo-inositol was synthesized and evaluated. The 5-deoxy-5-fluoro-inositol analogue is incorporated into cellular phospholipid and phosphorylated to a compound similar to PIP, but not further phosphorylated to the corresponding PIP2 derivative. This system is currently being used to evaluate analogues of bombesin.

It may be possible to alter the activity of an oncogene product by interfering with its localization in the plasma membrane. Myristoylation has

been shown to be critical for the membrane localization and cellular transforming activity of $p60^{src}$ and has been implicated for other transforming proteins. N-Myristoyl transferase has been further purified from bovine brain for the purpose of preparing antibodies to be used to clone the structural gene from brain libraries. A screen of newly synthesized compounds have identified two (i.e. 1-S-CoA-2-ketopentadecane and 2-bromotetradecanoyl-S-CoA) as *in vitro* inhibitors of N-myristoyl transferase activities.

These compounds are currently being examined in the Program's AIDS screen and in cell culture in our laboratory for their effect on $p60^{src}$ N-myristoylation. N-Myristoyl- and non-myristoyl- peptide homologous to the N-terminus of $p60^{src}$ have been used to identify a high affinity (D_d = 2.7 nM) protein "acceptor" in red cell membrane vesicles. Characterization of this "acceptor" suggests that it may be responsible for the specific localization of *src* to the plasma membrane/skeleton interface and may represent a new regulatory component for the $p60^{src}$ mediated tyrosine kinase activity involved in the normal cellular regulation and transforming activity of *c-src* and *v-src*, respectively.

N-Myristoylation also appears to be a critical event in the replicative cycle of mammalian retroviruses. Both in the case of type C viruses which assemble at the plasma membrane and type D viruses which bind the inner plasma membrane following assembly in the cytoplasm, the respective N-myristoylated *gag* gene products are apparently necessary for membrane binding and subsequent viral maturation. In both cases it has been shown that blocking *gag* N-myristoylation blocks viral development. Thus, N-myristoylation represents a unique new target for anti-AIDS drug development. A newly developed method of identifying N-myristoylated proteins which we recently developed has been used to unequivocally establish $p17^{gag}$ of HIV as an N-myristoylated protein.

Investigations were continued in two interrelated projects that center around the role of protein phosphorylation in various regulatory systems. The first project deals with the role of the proto-oncogene tyrosine protein kinase, c-*fes*, in myeloid cell differentiation. Two major studies were published by our laboratory this year dealing with the c-fes tyrosine kinase. The first study established that the previously purified and characterized 93 kDa tyrosine kinase of differentiated HL-60 cells was the gene product of c-fes, and that this activity was associated with more highly differentiated myeloid leukemias as well as with normal monocytes and granulocytes. The second study established that c-fes was an essential component of myeloid differentiation. The poorly differentiated myeloid leukemia cell line K562 does not express c-fes, but upon transfection with the c-fes gene acquired phenotypic characteristics of more mature cells as well as the ability to respond to a differentiation agent. The study is the first demonstration that a normal proto-oncogene is a prerequisite for myeloid differentiation. The second project has led to an interesting characterization of the proteolytic processing of protein kinase C in Adriamycin-resistant HL-60 cells. Our initial paper demonstrated that resistant cells have an abundance of both protein kinase C and the catalytic fragment of protein kinase C, termed M-kinase, compared to wild type cells. Antibodies to protein kinase C removed the M-kinase from cell extracts

and inhibited M-kinase-dependent phosphorylation in vitro. We have now characterized the proteolytic processing of three isoforms of protein kinase C in resistant cells, and show that these isoforms are processed differently. In addition, we have discovered that the gamma isoform of protein kinase C is induced or stabilized by phorbol esters only in resistant cells. Since phorbol esters appear to enhance drug resistance as well as activate protein kinase C, it appears that expression of specific isoforms of protein kinase C may be important in this phenomenon.

Studies were extended on the ADP-ribosylation factor of adenylate cyclase (ARF), a recently characterized G-protein. We have used a combination of biochemical, genetic, and immunological techniques to demonstrate that ARF is an essential gene found in every eukaryotic organism tested including man, yeast, slime mold, and plants. The ARF protein is structurally related to both the p21 ras oncogene family of small GTP-binding proteins as well as the G-protein family of membrane regulatory proteins. Microinjection of ARF into Xenopus, oocytes was shown to inhibit the p21 ras dependent or insulin stimulated meiotic maturation. Further efforts are underway to determine the extent of functional cross-reactivity of ARF with the ras proteins. ARF was shown to be both physically and functionally associated with Golgi membranes where it is highly concentrated on the cytosolic side of Golgi cisternae and appears to function as a regulator of proper protein processing. At least 7 ARF genes have been cloned and sequenced from four organisms including man, cow, yeast, and drosophila providing a good picture of the domains of the protein that have been absolutely conserved through evolution. We have expressed most of these proteins as well as a number of activated mutant forms of ARF in bacteria to allow the purification of mg amounts of protein for subsequent biochemical analyses. A detailed analysis of the nucleotide binding and hydrolysis properties of recombinant ARF has revealed a number of interesting features of the regulation of ARF activity.

We have discovered that a nuclear protein with a molecular weight of about 55,000 is retinoylated in HL60 cells. This is a new biochemical reaction discovered in this laboratory. The chemical properties of the retinoylated covalent bond indicates that it is a thioester linkage. Thus, retinoic acid is bound most likely to cysteine. Other cell lines, including HL60 mutants, have been examined for retinoylation. Qualitatively, the extent of retinoylation corresponds to the sensitivity of the HL60 cell line to differentiation by retinoic acid. All of the other cell lines had 3 or 4 retinoylated proteins. One of these proteins, common to all the cell lines, appears to be similar to the HL60 retinoylated protein. These results are consistent with an involvement of retinoylation in the response of cells to retinoic acid.

Flow cytometric techniques were developed to measure c-myc protein in individual HL60 cells. With fluorescence microscopy, c-myc protein was localized in the nuclei and was removed by treatment with DNase but not with RNase. In exponentially-growing cells the amount of c-myc protein/cell, normalized to cell volume, was constant throughout the cell cycle. During induction of differentiation with either DMSO or retinoic acid, decreases in c-myc protein/cell were correlated with decreases in cell growth and

the appearance of differentiated cells. It was not correlated with the known decreases in c-myc mRNA.

Rat kidney NADPH-linked high-Km aldehyde reductase was shown to be a potent inducer of monocytic differentiation of HL60 cells. The enzyme activity was not required for differentiation activity and it therefore appears that the inducing activity is related to the primary structure of the protein. Aldehyde reductase was shown to bind to specific surface receptors on HL60. There are about 13,000 receptors per HL60 cell. Attempts to sequence aldehyde reductase were not successful because of a blocked N-terminus.

A new project was initiated to study density dependent regulation of metabolic pathways. HMG-CoA reductase was studied in glial tumor cells at various cell densities. The results show that although enzyme activity and level declines continuously from sparse to confluent densities, RNA levels only begin to change when cells are at relatively high density. Different modes of regulation may correspond to differences in cell cycle controls over glial tumor cell growth rate that we reported previously.

A project involving traditional targets for drug design and study is a continuing project to determine the relative dependency of host and tumorous tissue on de novo vs salvage pathways for the synthesis of pyrimidine and purine nucleotides in vivo. De novo pyrimidine synthesis was studied in vivo by infusing mice with L-[^{15}N]alanine and measuring by mass spectrometry the incorporation of label into the uracil nucleotide pool of liver and intestine. From this data and the measured size of the uracil nucleotide pool, the absolute amount of uracil nucleotides formed by de novo synthesis during the infusion could be calculated. The amount of uracil nucleotides formed by de novo synthesis was linear in time for at least two hours, with the slopes giving an estimate of 29 $\pm$ 2 nmoles/gram/hour for the rate of de novo pyrimidine synthesis in liver, and 71 $\pm$ 4 nmoles/hour/gram in intestine. These results demonstrate the utility of L-[^{15}N] alanine infusions in the study of de novo pyrimidine synthesis in mouse liver and intestine.

The antitumor agent (NSC 368390, DuP 785) presently in clinical trials has been shown to inhibit the de novo pyrimidine mitochondrial enzyme dihydro-orotate dehydrogenase. In order to study the effects of this compound in intact cells we developed a GC/MS method to quantify dihydroorotate (DHO) and orotate (ORO). L1210 cells exposed to 2 uM NSC 36890 resulted in a net positive growth, increasing the doubling time to 120 h compared to 12 h control cells. The UTP pools in L1210 cells (0.2×10^5 - 5×10^5 cells/ml) continuously exposed to 2 uM NSC 368390 were reduced to less than 10% of control cell UTP levels. DHO in the treated cells continued to accumulate at a linear rate (8.3 +/-1.1 s.d. nmol/10^6 cells/h) over a time period of 10-70 h. Growth inhibition was prevented by simultaneous addition of uridine and NSC 368390, however, the addition of uridine after 48 h exposure of cells to NSC 368390 did not restore normal growth and soft agar reculture of these treated cells indicated <1% of the cell population was capable of colony formation. Quantification of DHO and UTP pools in treated cells after 48 h addition of uridine showed that DHO stopped

accumulating and that UTP levels were restored. The results of these studies suggests that cells treated with NSC 368390 are metabolically active but have been altered by a secondary effect of the compound which prevents cell division. Further studies are continuing to elucidate the mechanism of this secondary effect of NSC 368390 on cells.

Analysis of the characteristics of tryptophan antimetabolites on cell culture of L1210 murine leukemia cells revealed a unique feature of 4-fluoro-tryptophan not shared by its 5- and 6- positional isomers. The 4- isomer was still cytotoxic at concentrations equivalent to its metabolite, tryptophan and at even higher metabolite: antimetabolite ratios where the cytotoxicity of the isomers was abolished. Cytotoxicity was accompanied by unequal growth, failure to divide and subsequent lysis. The activity of the antimetabolite in the presence of its metabolite should make it effective in vivo where natural amino acid concentration cannot be controlled. Such studies with continuous release pellets established limit levels for host (mouse) toxicity. Reports that the cytotoxicity of interferon-gamma is due to tryptophan deficiency brought about by induction of the tryptophan degrading enzyme, indolemamine 2,3-dioxygenase has prompted studies on combination chemotherapy with the biological response modifier and antimetabolite.

The preceding summary outlines the objectives of the laboratory of Biological Chemistry and describes some of the research carried out within the Laboratory during the past year. The individual Project Reports, which follow, describe this research in greater detail.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06163-05 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacologic Aspects of Nucleotide Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R	Cysyk	Pharmacologist	LBC NCI
Others:	L	Anderson	Chemist	LBC NCI
	C	Chisena	Biologist	LBC NCI
	J	Kasofsky	IRTA Fellow	LBC NCI
	N	Malinowski	Chemist	LBC NCI
	J	Strong	Pharmacologist	LBC NCI
	D	Zaharevitz	Sr. Staff Fellow	LBC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The overall goal of this Project is to determine the relative importance of the de novo and salvage pathways for the synthesis of pyrimidine nucleotides in normal and malignant tissues in vivo, i.e., in the intact animal. This can be accomplished because of two recent advances in our Laboratory: (a) development of GC/MS methodology and a novel method of data analysis to quantitate pyrimidine de novo pathway activity and (b) discovery of an inhibitor of uridine kinase that is an effective inhibitor of pyrimidine salvage in vivo. These two tools will be used to assess the de novo and salvage pathways as targets for future antitumor drug development, as well as the therapeutic value of concurrent inhibition of both pathways.

De novo pyrimidine synthesis was studied in vivo by infusing mice with L-[^{15}N]alanine and measuring by mass spectrometry the incorporation of label into the uracil nucleotide pool of liver and intestine. From this data and the measured size of the uracil nucleotide pool, the absolute amount of uracil nucleotides formed by de novo synthesis was linear in time for at least two hours, with the slopes giving an estimate of 29+ 2 nmoles/gram/hour for the rate in liver, and 71+ 4 nmoles/hour/gram in intestine. The antitumor agent DUP-785 presently in clinical trials, was shown to inhibit the de novo pyrimidine mitochondrial enzyme dihydroorotate dehydrogenase. In order to study the effects of this compound in intact cells we developed a GC/MS method to quantify dihydroorotate (DHO) and orotate (ORO). The data from these studies show that cells treated with DUP-785 are metabolically active but are unable to resume cell division after removal of the drug. The mechanism of this secondary effect of DUP-785 is under investigation.

Objective:

The overall objective of this project is to determine the relative dependency of host and tumorous tissues on de novo vs salvage pathways for the synthesis of pyrimidine and purine nucleotides in vivo. The de novo biosynthetic pathways supply pyrimidines and purines for nucleic acid synthesis and are therefore considered to be important pathways for cell proliferation. Therefore, there has been a considerable effort throughout the past several decades to develop specific inhibitors of enzymes of these pathways. Although a number of potent inhibitors (e.g. PALA, pyrazofurin, 6-azauridine) exhibit excellent in vitro activity against isolated enzymes and cultured cells and in vivo activity against certain murine tumors, only marginal clinical success has been achieved with these agents. It would appear that factors other than potency of inhibition are important because very potent enzyme inhibitors (PALA, pyrazofurin) are only marginally effective. Accordingly, lack of clinical success with the pathway inhibitors developed to date is an indication that the importance of the de novo pathway to cell survival in vivo has been overestimated. If so, then the development of additional inhibitors of the de novo pathway would be a futile effort unless there is a coordinate development of agents that either interfere with the salvage pathway or with the synthesis and export of preformed nucleosides by donor organ(s).

This Project is divided into the following specific aims: to determine the physiologic importance of circulating pyrimidines and purines and their role in modulating the antitumor activity of antipyrimidine and antipurine chemotherapeutic agents; to study the liver as a modulator of circulating nucleosides and as a possible target for chemical manipulation; to develop agents to interfere with nucleoside salvage to be used in combination with inhibitors of de novo synthesis; to develop methodology for monitoring and quantitating the flux through the de novo pathways of host and tumorous tissues in vivo.

Major Findings:

Quantitation of pyrimidine synthesis de novo in the intact animal. We developed a method which utilizes ^{15}N-labeled precursors in conjunction with GC/MS and a novel method of data analysis to quantitate the actual amount of product formed by the pyrimidine de novo pathway in isolated rat hepatocytes. An important advantage of this method is that it can account for the amount of product formed by the pyrimidine de novo pathway that is not labeled. Also, values for the enrichment of pathway precursors are not required for the calculation and, in fact, this method allows the enrichment of the precursor pool to be determined; an important advance for the study of cellular compartmentation of metabolic pathways. We are currently applying this method in the intact animal. De novo pyrimidine synthesis was studied in mouse liver, intestine, and kidney by intraperitoneal infusion of $^{15}NH_4Cl$ and analysis of ^{15}N incorporation into uracil nucleotide pools. When the dose of a 1-h infusion if NH_4Cl was increased from 50μmol to 250μmol the fraction of the total uracil nucleotide pool formed by de novo synthesis increased 4.0-fold in liver to 8.4% and 2.3-fold in intestine to 13.7%. The increase in intestine was independent of the increase in liver as evidenced by the lack if

correlation between the increase observed in the intestine and liver of the same animal and the different distributions of label in the uracil ring nitrogens. A 2.4-fold increase in newly formed uracil nucleotides was observed in kidney when the infusion dose was raised from 150 μmol to 250 μmol. The increase in kidney was correlated with the increase in liver in the same animal and the distribution of label in the uracil ring nitrogens was similar to the distribution in liver. These results suggest that the increase in newly formed uracil nucleotides in intestine is due to increased de novo synthesis of pyrimidines in the intestines, while the increase in the kidney is due to increased salvage synthesis of uracil nucleotides from uridine synthesized in the liver and output to the circulation. De novo pyrimidine synthesis was studied in vivo by infusing mice with L-[^{15}N]alanine. The absolute amount of uracil nucleotides formed by de novo synthesis was found to be independent of the rate of infusion of L-[^{15}N]alanine (range of 2 mmoles/kg/hour to 12 mmoles/kg/hour) and was not changed when DL-[^{15}N]alanine or L-[^{15}N]alanine plus [^{15}N]glutamine was infused. The calculated amount of de novo pyrimidine synthesis also was not changed when the infusion was started one half hour after administration of uridine phosphorylase, an effective inhibitor of uridine salvage. The amount of uracil nucleotides formed by de novo synthesis was linear in time for at least two hours, with the slopes giving an estimate of 29 + 2 nmoles/gram/hour for the rate of de novo pyrimidine synthesis in liver, and 71 + 4 nmoles/hour/gram in intestine. These results demonstrate the utility of L-[^{15}N]alanine infusions in the study of de novo pyrimidine synthesis in mouse liver and intestine. The application of the method to the study of de novo pyrimidine synthesis in tumor tissues will be pursued.

Pharmacology of antipyrimidines: The antitumor agent (NSC 368390, DUP 785) presently in clinical trials has been shown to inhibit the de novo pyrimidine mitochondrial enzyme dihydroorotate dehydrogenase. In order to study the effects of this compound in intact cells we developed a GC/MS method to quantify dihydroorotate (DHO) and orotate (ORO). L1210 cells exposed to 2 μM NSC 36890 resulted in a net positive growth, increasing the doubling time to 120 h compared to 12 h control cells. The UTP pools in L1210 cells (0.2×10^5 - 5×10^5 cells/ml) continuously exposed to 2 μM NSC 368390 were reduced to less than 10% of control cell UTP levels. DHO in the treated cells continued to accumulate at a linear rate (8.3 =/-1.1 s.d. nmol/10^6 cells/h) over a time period of 10-70 h. Growth inhibition was prevented by simultaneous addition of uridine and NSC 368390 did not restore normal growth and soft agar reculture of these treated cells indicated <1% of the cell population was capable of colony formation. Quantification of DHO and UTP pools in treated cells after 48 h addition of uridine showed that DHO stopped accumulating and that UTP levels were restored. The results of these studies suggest that cells treated with NSC 358590 are metabolically active but have been altered by a secondary effect of the compound which prevents cell division. Further studies are continuing to elucidate the mechanism of this secondary effect of NSC 368390 on cells.

Publications:

Zaharevitz DW, Napier EA, Anderson LW, Strong JM, Cysyk RL, Stimulation of de novo pyrimidine synthesis in liver and intestine by ammonium chloride infusion, Eur J Biochem 1988; 175:193-198.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06167-05 LBC

PERIOD COVERED

October 1, 1989 to September 30. 1989

TITLE OF PROJECT *(80 charactars or less Title must fit on one line between the borders.)*

Inhib. of Myristoylation-Dependent Cell. Transformation & Retroviral Replication

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

NONE

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
3	1	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The modification of onc-proteins with the fatty acid myristate is an early step associated with the transformation of normal to neoplastic cells and mammalian retroviral reproduction and it is thought to be part of the mechanism by which cytoplasmic oncogene protein kinases or viral gag structural proteins are localized to the inner plasma membrane surface. Since the transforming activity of onc-kinases and viral replication is dependent upon this membrane binding, this project will investigate the role of myristoylation as it relates to the mechanism of this subcellular localization and as a potential target for chemotherapeutic drug development. N-Myristoyl transferase has been further purified from bovine brain for the purpose of preparing antibodies to be used to select clones of the enzyme gene from brain libraries. A screen of newly synthesized compounds have identified two compounds (i.e. 1-S-CoA-2-ketopentadecane and 2-bromotetradecanoyl-S-CoA) as in vitro inhibitors of N-myristoyl transferase activity. These compounds are currently being examined in the Program AID's screen and in cell culture in our laboratory for their effect on HIV and RovS sarcoma virus replication. N-Myristoyl- and non-myristoyl- peptides homologous to the N-terminus of p60src have been used to identify a high affinity (k_d = 2.7 nM) protein "acceptor" in red cell membrane vesicles. Characterization of this "acceptor" suggests that it may be responsible for the specific localization of src to the plasma membrane/skeleton interface and may represent a new regulatory component for the p60src mediated tyrosine kinase activity involved in the normal cellular regulation in our laboratory and transforming activities of c-src and v-src, respectively. Using a new method recently developed in our laboratory we have unequivocally established p17gag of HIV as an N-myristoylated protein.

Tyrosine-specific protein kinase activity is associated with several known oncogenes and is an appealing target for the chemical manipulation of kinase associated cellular transformation. The transforming activity of onc-kinases depends upon their association with the inner plasma membrane surface. It has been proposed that the mechanism by which viral encoded onc-kinases such as p60src become membrane bound is through a co-translational addition of myristic acid to their NH2-terminal glycine via an amide linkage. Myristoylation is also an essential step in the replication of type B,C & D retroviruses and is typified by the covalent attachment of myristic acid to the NH2-terminal glycine of viral gag structural proteins via an amide linkage. These myristoylated gag proteins are then localized to the inner plasma membrane where viral assembly and maturation occurs. The importance of myristoylation to onc kinase transformation and retroviral replication has been illustrated by oligonucleotide-directed mutagenesis, resulting in mutant gene proteins in which NH2-terminal glycines were either absent or replaced with alanine. These mutant proteins no longer transformed cells nor were viral particles formed. Presumably, myristoylation is critical to onc-kinase transformation and retroviral reproduction.

Objectives.

In this project we will examine the role of myristoylation of onc-tyrosine kinases and retroviral gag structural proteins as targets for the chemotherapeutic inhibition of cellular transformation and viral reproduction. Specifically, we will study three mechanisms for blocking myristoylation dependent transformation by p60src: these include, (i) inhibition of the myristoyl transferase(s), (ii) inhibition of binding of myristoyl-proteins to potential membrane receptors; and (iii) inhibition of cytoplasmic of translocation of N-myristoyl proteins.

Major Findings.

1. Myristoyl Dependent High Affinity Binding of Peptides Homologous to the N-terminus of pp60src with Red Cell Membrane Vesicles. N-Myristoyl and non-myristoyl peptides corresponding to the N-terminus of p60src were used to examine whether N-myristoylation facilitates the binding of p60src to specific protein sites at the plasma membrane. We discovered high affinity protein acceptor sites (Kd = 2.7 nM) to a 15 amino acid N-myristoylated N-terminal p60src peptide in red cell membrane vesicles. Binding was not competed by the non-myristoylated analog of the peptide nor by shorter N-myristoyl src peptides and peptides homologous to the N-terminus of other N-myristoylated proteins. Binding was not evident after treatment of vesicles with proteolytic enzymes. Raising the salt concentration of the buffer to 50 mM NaCl caused an apparent inhibition of binding. However, no significanteffect of salt was observed on the off-rate of bound ligand under these conditions. The results indicate the existence of N-myristoyl dependent p60src protein acceptor sites at or near the plasma membrane/skeleton interface of red cells which could be responsible for the localization of p60src to this region and may represent new regulatory components for p60src mediated tyrosine kinase activity.

2. Identification of N-myristoylated Proteins by Reverse-phase High Performance Liquid Chromatography. The N-terminal p17gag protein of the human immunodeficiency virus has been shown to incorporate radioactivity following labelling of infected cell lines with [3H]myristic acid. We investigated p17gag to determine whether the incorporated radioactivity was the consequence of N-terminal myristoylation. The virus was purified by density gradient centrifugation after labelling chronically infected H9 cells with [3H] myristic acid. The $p17_{gag}$ was isolated by immunoprecipitation and subject to partial acid hydrolysis. [3H]Myristoylglycine generated by the hydrolysis was derivatized, to 4-(p-nitrobenzylidene)-2-tridecanoyloxazol-5-one and identified against a co-eluting, derivatized, unlabelled N-myristoylglycine standard by reverse-phase high performance liquid chromatography. This study unequivocally demonstrates that p17gag is an N-myristoylated protein.

3. N-Myristoylation of p60src; Identification of a myristoyl-CoA:glycylpeptide N-myristoyltransferase in rat tissues. A 16-residue synthetic peptide corresponding to the N-terminal sequence of p60src was used as the acyl acceptor in an assay for myristoyl-CoA:glycylpeptide N-myristoyltransferase in rat tissues. An additional C-terminal tyrosine amide was added to this peptide to facilitate radioiodination and enhance detectability. Reverse-phase h.p.l.c. enabled the simultaneous detection and qualification of the peptide substrate and its N-myristoylated product. N-Myristoyltransferase activity was highest in the brain with decreasing activities in lung, small intestine, kidney, heart, skeletal muscle and liver. Brain activity was distributed approximately equally between the 100000g pellet and supernatant fractions. The soluble enzyme exhibited a Kapp of 20 M for the src peptide and an optimum between pH 7.0 and 7.5. Maximum N-acylating activity was seen with myristoyl (C14:0)-CoA with lower activities found with the (C10:0)-CoA and (C12:0)-CoA homologues. No activity was obtained with palmitoyl (C18:0)-CoA but this derivative inhibited N-myristoyltransferase activity >50% at equimolar concentrations with myristoyl-CoA. With a decapeptide corresponding to the N-terminal sequence of the cyclic AMP-dependent protein kinase catalytic subunit as the acyl acceptor, the brain enzyme displayed a Kapp of 117 um and was about 14-fold less catalytically effective than with the p60src acyl acceptor. Transferase activity was also seen with a 16-residue peptide corresponding to the N-terminal sequence of the HIV p17gag structural protein. Inhibition studies with shorter src peptide analogues indicated an enzyme specificity for the p60src acyl acceptor beyond 9 residues.

Proposed Course.

Myristoylation is thought to be an important cellular phenomenon. The fact that such a rare fatty acid is utilized with such absolute specificity by cellular enzymes with broad regulatory effects as well as by transforming onc-kinases suggest it has a central role in the control of cellular growth and differentiation. Its involvement in mammalian retrovirus replication utilizes the basic myristoylation pathways. The work in this project is designed to clarify several specific aspects concerning the process of myristoylation. From these studies, it may be possible to design novel new

compounds for specifically blocking myristoylation. These agents may then provide us with pharmacologic tools to specifically inhibit myristoylation dependent cellular transformation and mammalian retroviral related disorders. At least three key aspects of myristoylation dependent membrane binding of transforming oncogene and retroviral gag structural proteins may be susceptible to direct chemotherapeutic manipulation. These include, (1) inhibition of the myristoyl transferase(s), (ii) inhibition of binding of myristoyl-proteins to potential membrane receptors and (iii) inhibition of cytoplasmic translocation of N-myristoyl proteins. Little is known about N-myristoyl transferase(s) from mammalian sources nor is there any information about hypothetical N-myristoyl protein membrane receptors and cytoplasmic translocation. We would predict that all three steps are essential to the overall myristic acid dependent transforming and viral maturation mechanisms and therefore, each represents an opportunity to block malignancy that results from this type of transformation or inhibit retroviral reproduction.

(i) N-Myristoyl Transferase(s). We are continuing our characterization of mammalian myristoyl transferases using peptide acceptors corresponding to the NH2-terminal sequence of p60src, p24gag and cAMP dependent protein kinase. The enzyme(s) is being purified from bovine brain and the enzymatic mechanism characterized. We have designed several new possible, irreversible and competitive inhibitors. These have been synthesized and are now being tested as inhibitors of the enzyme. Their ability to inhibit oncogene-kinase mediated transformation and retroviral replication in tissue culture cells is also being examined.

(ii) N-Myristoyl Protein Membrane Binding. Membrane binding sites for p60src N-myristoyl-protein have been described in red cells. We will also look for similar binding sites in platelets and transformed cell lines. N-myristoyl protein receptors will be characterized and purified by affinity absorption to peptide affinity resins. Specific agents will be designed to block N-myristoyl-protein receptor association. These will be tested as inhibitors of ocogene-kinase mediated transformation and/or retroviral replication in tissue culture cells.

(iii) Myristoyl-protein translocation. Shortly after synthesis on free ribosomes, the myristoyl-p60src kinase forms a soluble complex with two cellular proteins of 50 and 90 kilodaltons. It has been suggested that this complex may act as a device for transporting newly synthesized p60src kinase to its site of transforming action at the plasma membrane. Similar complexes have been reported for onc-kinases encoded by other retroviruses so "complex mediated" translocation might be another important step in onc-kinase cellular transformation.

Publications.

Beck WT, Cirtain MC, Glover CJ, Felsted RL, Safa AR. Effects of indol alkaloids on multidrug resistance and labeling of P-glycoprotein by a photoaffinity analog of vinblastine, Biochem Biophys Res Comm 1980; 153: 959-966.

Goddard C, Felsted RL, Identification of N-myristoylated proteins by reverse-phase high performance liquid chromatography of an azlactone derivative of N-myristoylglycine, Biochem J 1988; 253:839-843.

Felsted RL, Goddard C, Glover CJ, N-Myristoylation as a novel molecular target for the design of chemotherapeutic drugs, Glazer RI, (Ed), Developements in Cancer Chemotherapy, Boca Raton FL, CRC Press 1989; 95-115.
Goddard C, Angelo A, Glazer R, Felsted RL, Chemical characterization of $P17^{gag}$ from HIV as an N-terminally myristoylated protein, Euro J Biochem 1989; in press.

Goddard C, Arnold ST, Felsted RL, High affinity binding of an N-terminal myristoylated p60src peptide, J Biol Chem 1989; in press.

McGrath T, Latoud C, Arnold ST, Safa AR, Felsted RL, Center MS, Mechanisms of multidrug resistance in HL60 cells; analysis of resistance associated membrane proteins and levels of mdr gene expression, Cancer Res 1989; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06179-04 LBC

PERIOD COVERED
October 1, 1988 to Sepemeber 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Proto-Oncogene Tyrosine Protein Kinase Activity in Myeloid Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Laboratory of Biological Chemistry

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The aim of this project is to characterize the role of proto-oncogene tyrosine protein kinase activities in myeloid differentiation. These studies have centered on the relationship between the expression of the proto-oncogene tyrosine protein kinase, c-fes, and myeloid differentiation in various myelomonocytic cell lines. This has been investigated by protein blotting with antibodies to specific antigenic determinants of $p93^{c\text{-}fes}$, by determining the effects of tyrosine protein kinase inhibitors on differentiation in sensitive and resistant cells, by transfecting K562 cells (which do not express c-fes) with the c-fes gene, and by investigating the transcriptional regulation of c-fes mRNA using an RNase protection assay. Future studies will be directed at the use of antisense oligodeoxynucleotides to the initiation codon of $p93^{c\text{-}fes}$ mRNA, transfecting HL-60 cells with the antisense sequence to the first coding exon, and by analyzing the role of transcriptional enhancer elements in the LTR of genomic c-fes. Also planned are studies to develop a transgenic mouse model for myelodysplastic disease by overexpression of the c-fes gene. The latter studies in particular, may give us a clue as to why myeloid leukemias display unregulated growth. In this regard, we have established a collaborative clinical study assessing the diagnostic and prognostic value of c-fes mRNA levels in patients with myeloid leukemias prior to and after developing resistance to chemotherapy.

Objectives.

The aim of this project is to characterize the role of the $p93^{c}$-fes proto-oncogene tyrosine protein kinase (PKT) in myeloid differentiation. Recently, we reported the purification and characterization of a 93 kDa PKT from DMSO-differentiated HL-60 leukemia cells. We further established the association of this enzyme with myelomonocytic differentiation and its absence in resistance to differentiation. We have now unequivocally identified the 93 kDa PKT as the gene product of the human c-fes locus and have established that its activity is closely coordinated with the maturation of myelomonocytic cells. We have further established the role of c-fes in myeloid differentiation by showing that differentiation-resistant myeloblast cells (K562 cells) transfected with genomic c-fes acquire the ability to undergo differentiation. We have further quantitated the c-fes mRNA levels in a variety of human myelomonocytic cell lines by the use of an RNase protection assay and have concluded that the expression of c-fes mRNA during differentiation is regulated at the transcriptional level. Future studies will be directed at the use of antisense oligodeoxynucleotides to the initiation codon of $p93^{c\text{-}fes}$ mRNA, transfecting HL-60 cells with the antisense sequence to the first coding exon, analyzing the role of transcriptional enhancer elements in the LTR of genomic c-fes, and by transfecting K562/c-fes cells with an isoform of protein kinase C to study whether this tyrosine protein kinase is regulated by serine/threonine phosphorylation. Also planned are studies to develop a transgenic mouse model for myelodysplastic disease by overexpression of the c-fes gene. The latter studies in particular, may give us a clue as to why myeloid leukemias display unregulated growth. In this regard, we have established a collaborative clinical study assessing the diagnostic and prognostic value of determining the expression of c-fes mRNA in patients with myeloid leukemias at presentation and after developing resistance to chemotherapy.

Significance.

The regulation of viral oncogene and proto-oncogene expression is an area of investigation which should lead to a clearer understanding of viral transformation as well as normal cellular proliferation and differentiation. Among the 20 viral oncogenes detected thus far, approximately 50% produce a PKT activity as their gene product. An equal number of PKT activities have been detected in normal tissues and there is a close homology between the viral oncogene PKT and the normal cellular enzyme in many instances. PKT activity is associated with the membrane receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, insulin and macrophage colony stimulating factor (M-CSF). In the latter case, the PKT is the gene product of the c-fms proto-oncogene. This enzymatic activity provides a functional relationship between many growth factors and proto-oncogene products and thus, undoubtedly plays a central role in the signal transduction process. In a similar context, there is evidence that PKT activity may play a role in differentiation. The dependence on M-CSF for macrophage colony formation from normal chicken macrophage progenitor cells was abrogated in v-fps-infected cells which formed macrophage colonies independently of colony stimulating factors. The v-fps oncogene is the avian

counterpart of v-fes. The expression of proto-oncogene c-fms mRNA was increased during differentiation of promyelocytic leukemia cell line HL-60 to the macrophage phenotype by the phorbol ester TPA. The c-fms gene product was shown to be a 140-170 kDa PKT activity and it appears to be the receptor for M-CSF. Differentiation of HL-60 cells to the granulocytic or monocytic/macrophage phenotypes by DMSO or TPA, respectively, resulted in the increased appearance of $pp60^{c-src}$ PKT. PKT activity was also demonstrated in the particulate fraction of the monocytic cell line U-937 and to be stimulated in vitro by TPA. Uncharacterized PKT activities which increase slightly during granulocytic or macrophage differentiation of HL-60 cells have also been reported. A new hematopoietic cell kinase (hck) gene that is especially prominent in cells of the myeloid lineage and codes for a 57 kDa product similar to $pp60^{c-src}$ was recently reported by two laboratories. The expression of the c-fes gene product is also associated with cells of the myeloid lineage and contains PKT activity. Our laboratory recently reported the identification of p60 and p93 PKT activities in HL-60 cells, the latter of which was associated with granulocytic or monocytic differentiation. p93 was antigenically related to residues 739-768 and 790-805 of the v-fes gene product, a region adjacent to the autophosphorylation site of this protein. We have now demonstrated using polyclonal antibodies against various domains of the cloned c-fes protein that p93 is the c-fes proto-oncogene product. This study was the first reported purification of a human proto-oncogene product with PKT activity. Our laboratory has also demonstrated for the first time the role of a human proto-oncogene in myeloid differentiation.

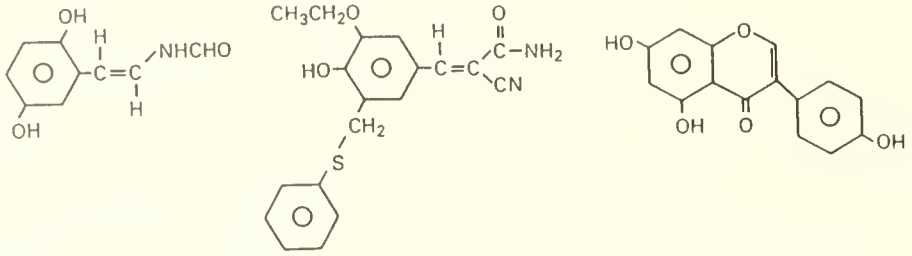
Major Findings.

A differentiation-associated 93 kDa tyrosine kinase (p93) was purified previously from the human promyelocytic leukemia cell line HL-60. The present study conclusively identifies p93 as the c-fes proto-oncogene product, and shows that expression of $p93^{c-fes}$ and its associated tyrosine kinase activity are marked in mature granulocytes, monocytes, and human myeloid leukemia cell lines. Antisera to peptides obtained by expression of c-fes cDNA fragments in E. coli reacted strongly with p93 purified from HL-60 cells. Western blots using one of these antisera demonstrated high levels of $p93^{c-fes}$ protein in normal human granulocytes and monocytes, as well as the cell lines KG-1, THP-1, HEL, and U-937, all of which can be induced to differentiate along the myelomonocytic pathway. Conversely, in cell lines resistant to myeloid differentiation, $p93^{c-fes}$ expression was either very low or absent. Expression of immunoreactive $p93^{c-fes}$ in these cell lines showed a strong positive correlation with $p93^{c-fes}$ tyrosine kinase activity, which was measured in cell extracts using a non-denaturing gel assay. Finally, the expression of $p93^{c-fes}$, its tyrosine kinase activity, and the binding of [^{125}I]granulocyte-macrophage colony-stimulating factor (GM-CSF) were all coordinately increased in HL-60 cells treated with the granulocytic differentiation inducer dimethylsulfoxide, while all three parameters were low in untreated or differentiation-resistant HL-60 cells. These results suggest that expression of $p93^{c-fes}$ tyrosine kinase activity may be an essential component of myeloid differentiation and responsiveness to GM-CSF.

A subsequent study examined whether cells that neither underwent differentiation nor expressed c-fes could acquire the ability to differentiate following transfection with the c-fes gene. The K562 cell line is resistant to myeloid differentiation and does not express c-fes. Upon transfection and clonal selection of K562 cells using a mammalian expression vector containing the 13 kb human c-fes gene, c-fes mRNA was transcribed and $p93^{c\text{-}fes}$ tyrosine activity kinase was expressed. Clones expressing c-fes underwent myeloid differentiation as assessed by the appearance of phagocytic activity, Fc receptors, NBT reduction, Mac-1 immunofluorescence, and lysozyme production. These results indicate that the expression of the c-fes proto-oncogene and its associated tyrosine kinase activity play a major role in the initiation of myeloid differentiation.

A collateral study examined c-fes mRNA levels in a panel of human leukemia cell lines previously shown to exhibit a positive correlation between responsiveness to differentiation inducers and $p93^{c\text{-}fes}$ tyrosine kinase activity. Using an anti-sense RNA probe complementary to exon 2 of the human c-fes sequence, RNase protection assays of poly(A)-RNA isolated from the differentiation-competent cell lines HL-60, KG-1, HEL, THP-1, and U-937 revealed a major band 222 nucleotides in length, which corresponds to c-fes exon 2. Minor bands of 320 and 370 nucleotides were also observed, suggesting that alternate 5' splicing of the c-fes transcript occurs. By contrast, the differentiation-resistant variant KG-1a expressed very little c-fes mRNA, and no c-fes transcripts were detected in K-562 erythroleukemia cells. Four-day treatment of HL-60 promyelocytes with the granulocytic inducer Me_2SO resulted in a two-fold increase in c-fes mRNA and protein levels. Increases in c-fes transcripts were apparent after 24 hours of Me_2SO exposure and reached a plateau at 72 hours; this time course parallels the appearance of differentiated cells and $p93^{c\text{-}fes}$ tyrosine kinase activity. Nuclear run-off assays showed that the Me_2SO-induced increase in HL-60 c-fes mRNA is due to enhanced transcriptional activity. These data suggest that regulation of c-fes expression in myelomonocytic cells occurs at the transcriptional level, and that the increase in $p93^{c\text{-}fes}$ tyrosine kinase activity associated with granulocytic differentiation of HL-60 cells is due, in part, to enhanced transcription of the c-fes gene.

We have also assessed several tyrosine kinase inhibitors in HL-60 and DMSO-resistant HL-60 (HL-60/DMSO) cells. Cells were treated with the PKT inhibitors, erbstatin, genistein and ST638 continuously for 1 to 3 days. Differentiation was determined by nitroblue tetrazolium (NBT) reduction and viability by trypan blue exclusion. HL-60/DMSO cells, which lack c-fes, were resistant to the cytotoxicity produced all three drugs. In contrast, the viability of HL-60 cells was reduced by 35% at 25 μM erbstatin, by 45% at 50 μM genistein and by 60% at 50 μM ST638 after 3 days of treatment. Among the three PKT inhibitors, only erbstatin and genistein induced NBT positive cells after this treatment interval. Erbstatin at 10 and 25 μM produced 45 to 50% NBT positive cells, and 10 μM genistein produced 45% NBT positive cells. Thus, there appeared to be a correlation between the expression of c-fes and the cytotoxicity produced by the PKT inhibitors, but there ability to induce differentiation did not relate to cytotoxicity. Indeed, the fact that a PKT



ERBSTATIN ST638 GENISTEIN

Figure 1. Structures of tyrosine protein kinase inhibitors.

inhibitor would induce differentiation at all is an anomaly since c-fes tyrosine kinase activity is a prerequisite for differentiation. One can only conclude that these inhibitors are acting on other cellular targets beside c-fes. One possibility is that they are interfering with topoisomerase activity. That PKT inhibitors do inhibit topoisomerase activity has recently been reported. The mechanism may have to do with the tyrosine phosphate intermediate as a result of the association of the topoisomerase with the 5'-terminal phosphate of the cleaved DNA. This would be an interesting area to explore for chemotherapeutic drugs which may also have the ability to induce differentiation.

Experimental Design and Methods.

HL-60, U-937, THP-1, KG-1, KG-1a, and COS-1 cell lines were obtained from the American Type Culture Collection. HL-60/DMSO and HL-60/TPA cells were provided by Dr. Steven Grant, Medical College of Virginia. Human peripheral blood monocytes and granulocytes were obtained from the NIH Blood Center. Cell culture conditions and cytochemical determinations of differentiation have been described previously.

Tyrosine kinase inhibitors that are currently available are genistein (Extrasynthese, France), erbstatin (Drug Synthesis and Chemistry Branch, DTP, NCI) and ST-638 (Kanegafuchi Chemical Industry Co., Japan).

Tissue culture conditions for inducing differentiation of HL-60 cells have been described. The conditions for the other myeloid cell lines will be determined on an individual basis. Cells will be used following induction of differentiation with DMSO or IFN- (HL-60 cells), GM-CSF, IFN- and $1,25(OH)_2$vitamin D_3 (U-937), or retinoic acid (THP-1) for the appropriate time interval required for maximum induction of NBT positive cells (granulocytic and monocytic cells) and nonspecific esterase (monocytic cells). Fc receptors will be determined visually by microscopic examination using anti-sheep red blood cell coated sheep erythrocytes and erythrophagocytosis will be determined under similar conditions after lysing extracellular erythrocytes. Macrophage-like cells will be characterized by their morphology and adherence to the plastic flask. KG-1 (GM-CSF-sensitive)

and KG-1a (GM-CSF-resistant) cells will be used after treatment with GM-CSF.

Cells will be extracted with Triton X-100 in a buffer containing protease inhibitors and sodium vanadate to inhibit phosphotyrosine phosphatase.

Cloning of $p93^{c}$-fes will employ the 13 kb Eco RI genomic sequence of c-fes (American Type Culture Collection) cloned into the expression vector pECE which has been used to express the insulin receptor tyrosine kinase. This vector was obtained from Dr. William J. Rutter, University of California San Francisco. Insertion of the Eco RI c-fes fragment into this vector is simple since pECE contains an Eco RI restriction site in the polylinker region. However, for selection of stable transformants, cotransfection with pSV2NEO (Clontech) is necessary followed by selection in G418.

Transient expression will be carried out with COS-1 cells in order to test whether or not the c-fes constructs can be transcribed to yield $p93^{c}$-fes. The expression of p93 was determined by Western blotting, autophosphorylation, and non-denaturing gel assays. Stable expression of the c-fes vector was performed using the protoplast fusion technique. Cells successfully transfected with either pECE/fes + pSV2NEO were selected for resistance to the neomycin analog G418 (Gibco) since the neo gene codes for a phosphotransferase with inactivates the antibiotic. HL-60 cells will be transfected with the antisense construct of exon 2 of c-fes to determine if myeloid differentiation can be blocked at the transcriptional level. Stable transfected cells will be examined for their ability to undergo differentiation in response to specific differentiating agents such as TPA.

Oligodeoxynucleotides against the initiation region of the c-fes mRNA will be synthesized using the individual blocked deoxynucleotide phosphothioates (Synthecell). The 18-mer antisense construct will be directed against the start codon and adjacent sequences downstream from exon 2 of c-fes. This sequence is: 5'-ATG GGC TTC TCT TCC GAG, and thus, the antisense sequence is 5'-CTC GGA AGA GAA GCC CAT. Assessment of the activity of these compounds will be by RNase protection assay using an RNA probe encompassing exon 2 in either the antisense direction (for c-fes mRNA) or sense direction (for antisense c-fes mRNA).

Publications.

Yu G, Grant S, Glazer RI, Association of $p93^{c\text{-}fes}$ tyrosine protein kinase with granulocytic/monocytic differentiation and resistance to differentiating agents in HL-60 leukemia cells, Mol Pharmacol 1988; 33:384-388.

Smithgall TE, Yu G, Glazer RI, Identification of the differentiation-associated p93 tyrosine protein kinase of HL-60 leukemia cells as the product of the human c-fes locus and its expression in myelomonocytic cells, J Biol Chem 1988; 263:15050-15055.

Glazer RI, Differentiation of malignant cells as a strategy for cancer treatment, New Directions in Cancer Treatment, I Magrath (ed.), UICC-Springer-

Verlag, Heidelburg Germany 1988; 428-443.

Glazer RI, Aquino A, Yu G, Cellular oncogenes as biotherapeutic targets for the differentiation and inhibition of cancer cells, Developments in Cancer Chemotherapy, Volume II RI Glazer (ed.), CRC Press, Boca Raton Florida, 1-24.

Glazer RI, Cyclopentenyl nucleoside analogs as antiviral and anticancer drugs. Antiviral Drugs: Basic and Therapeutic Aspects of Antiviral Drugs, R Calio, G Nistico (eds.), Pythagora Press, Milan Italy 1989; 97-120.

Yu G, Smithgall TE, Glazer RI, K562 leukemia cells transfected with the human c-fes gene acquire the ability to undergo myeloid differentiation, J Biol Chem 1989; 264:10276-10281.

Goddard C, Aquino A, Glazer RI, Felsted RL, Chemical characterization of $p17^{gag}$ from human immunodeficiency virus (HIV) as an N-terminally myristoylated protein, Eur J Biochem, in press.

Chapekar MS, Knode MC, Glazer RI, The EGF- and interferon-independent effects of double-stranded RNA in A431 cells, Mol Pharmacol 1988; 34:461-466.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06181-04 LBC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interaction of GTP-binding Proteins with Cellular Components

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Richard A. Kahn Senior Staff Fellow LBC, NCI

Others: Cherrie Rulka Biologist LBC, NCI
Janet Holden Visiting Fellow LBC, NCI

COOPERATING UNITS *(if any)*

Mark Willingham IMBGY

LAB/BRANCH
Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An emerging family of structurally related proteins, the small GTP-binding proteins (SGBP), have been implicated as regulators of a diverse array of cellular functions; including cell transformation and growth, protein synthesis and processing, targeting of membrane vesicles and organelles, and activation of second messenger systems (e.g. phospholipase C). Members of the SGBP family include p21 ras, rho, ral, ypt, sec4, and ARF proteins. We have focused on the ADP-ribosylation factor (ARF) in these studies although it is likely that all of these proteins will share common themes of cellular regulation. Immunocytochemistry has allowed the demonstration of a high degree of localization of ARF in mammalian cells on the cytosolic side of Golgi membranes. Microinjection of ARF into Xenopus laevis oocytes has revealed a new possible role for ARF as an anti-oncogene. Injected ARF was shown to block the insulin or p21 ras induced maturation of oocytes.

We have recently completed a thorough analysis of the binding and hydrolysis of guanine nucleotides by ARF proteins and have demonstrated the central role of free metal and bound GDP in controlling activation of the protein. Part of this project included the purification of recombinant (bacterially expressed) ARF proteins for use as a reagent in subsequent studies. Analysis of the structure of different ARF proteins as well as engineered mutant ARF proteins has already yielded a fairly good idea of the functional domains of the protein. The identification and purification of proteins which regulate the hydrolysis and activation of the ARF proteins is underway and should yield further information on the extent of functional interaction between ARF and p21 ras proteins.

PHS 6040 (Rev. 1/84) GPO 914-918

Since the discovery that the oncogene p21 ras is a small GTP-binding protein there have been more than 30 related proteins cloned and sequenced which share some degree of structural similarity. The function of none of these other SGBPs is known. During this time the PI has identified, purified, cloned, and sequenced another SGBP, the ADP-ribosylation factor, ARF, based on its defined biochemical activity as cofactor in the cholera toxin dependent activation of adenylate cyclase. As ARF is the only SGBP which has been purified from mammalian tissues based on a biochemical assay, much more is known about its function than the others, yet I believe that the physiological role of ARF has yet to be defined. These studies are aimed at elucidating such a role(s) and defining the detailed biochemical steps in the regulation of cellular function by ARF as a prototype for the ras and ras-related proteins.

Objectives.

In all cases where a cellular role is known the activity of a guanine nucleotide binding protein is controlled by the binding of GTP. Hydrolysis of the bound GTP results in deactivation of the protein and pathway. Thus, knowledge of the factors which control the binding and hydrolysis of GTP will also identify elements upstream and downstream of the regulatory proteins. It is likely that the downstream component will be the effector which defines the endpoint of the pathway. The objective of this work is to utilize the nucleotide binding properties of ARF and identify cellular targets and pathways controlled by this protein. Once identified these proteins will be purified and analyzed to determine their role in ARF and cellular regulation. It is hoped that results with ARF may be directly relevant to p21 ras and related proteins.

Methods Employed.

We are using a combination of biochemical, genetic, and molecular biology techniques to address the question of the physiological role of ARF and identification of the components in the ARF pathway. This project focuses on the details of the structure of different ARF proteins both to define the functional domains of the protein as well as to aid in the construction of useful mutant forms of the protein. Toward this end we have cloned and sequenced two human ARF genes, one of two bovine genes, two yeast homologues, and we are collaborating on the drosophila ARF. The comparison of conserved sequence gives very good evidence of structurally important regions of the 181 amino acid protein. We have used this and related information to construct at least 6 deletion or point mutations.

Each of the proteins have been expressed in bacteria to produce large (mg) amounts of protein for analysis. In addition, the genes have been inserted into the yeast genome or on episomal plasmids to control the expression of the foreign genes in yeast; where we have identified a number of ARF dependent phenotypes. Thus, we can study the effects of specific mutations biochemically and in vivo. Biochemical analysis of factors which regulate the binding and hydrolysis of guanine nucleotides to ARF are performed at the same time that we are screening yeast libraries for genes which affect ARF action in yeast and

should yield related information. This combined approach is both more powerful and should greatly speed the cloning of relevant genes.

Major Findings.

The ability of ARF to block p21 ras dependent meiotic maturation of Xenopus oocytes has revealed a potential interaction of ARF with the oncogene and raised the possibility of using ARF to inhibit ras activity in mammalian cells. However, the frog oocyte system is extremely limited in what can be done so we have concentrated our efforts on the biochemical characterization of components in the ARF pathway and comparison to elements in the p21 ras pathway.

We have cloned human ARF and shown it to be identical to bovine ARF1. A second human ARF was sequenced and expressed in bacteria, as were both yeast ARF genes, a drosophila ARF, one deletion mutant of bovine ARF1, and 5 point mutants of yeast ARF1. We are just finishing the biochemical characterization of these proteins to determined the consensus sequences for each ARF activity (nucleotide binding, hydrolysis, ARF activity, membrane localization, Golgi localization). An in depth analysis of the nucleotide binding characteristics of recombinant bovine ARF1 has been submitted for publication and contains new information on the role of bound GDP and free magnesium in controlling the rate of activation of ARF. Finally, we have identified a protein in yeast cytosol which is capable of turning on the intrinsic GTPase activity of yeast ARF. We are currently analyzing the relationship of this protein to ras GAP and other regulatory proteins.

Publications.

1. Kahn RA, Goddard C, Newkirk M, Chemical and Immunological Characterization of the 21 kDa ADP-ribosylation Factor (ARF) of Adenylate Cyclase, J Biol Chem 1988; 263:8282-8287.

2. Sewell JL, Kahn RA, Sequences of the bovine and yeast ADP-ribosylation factor and comparison to other GTP-binding proteins, Proc Natl Acad Sci USA, 1988 85:4620-4624

3. Botstein D, Segev N, Stearns T, Hoyt MA, Holden J, Kahn RA, Diverse Biological Functions of Small GTP-Binding Proteins in Yeast, CSH Sympos Quant Biol 1988, 53:629-636.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06182-04 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic and Immunologic Analyses of the ADP-ribosylation Factor, ARF

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard A. Kahn	Senior Staff Fellow	LBC, NCI
Others:	Jenny Sewell	Microbiologist	LBC, NCI
	Ofra Weiss	Visiting Fellow	LBC, NCI

COOPERATING UNITS *(if any)*

None

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Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

ARF is a recently characterized GTP-binding regulatory protein component of the adenylate cyclase system. These studies are aimed at the elucidation of the role of ARF in signal transduction, differentiation, and proliferation. We have used the bovine ARF gene to clone two homologous genes, ARF1 and ARF2, from the yeast, S. Cerevisiae. Disruption of ARF1 leads to slow growth, cold sensitivity, and supersensitivity to fluoride. We plan to investigate the consequences of ARF2 disruption both alone and in combination with disruption of ARF1. Suppressors of the arf1- phenotype have been obtained and will be analyzed. It is expected that some of these suppressor genes will code for other proteins in the ARF pathway. We will determine the ability of human ARF to rescue the arf1- phenotype to test for conservation f ARF function. In addition, we are cloning and sequencing ARF from both human and Xenopus laevis libraries. The human gene will be used to screen a variety of human tumors for the presence of abnormal ARF expression. The Xenopus gene will be used in studies of ARF function in Xenopus oocytes which allow microinjection of purified ARF proteins and biochemical assays of resultant changes in cellular metabolism.

Introduction

ARF has recently been identified as a 21,000 Da GTP-binding regulatory protein. Activated ARF binds to the stimulatory, regulatory component (Gs) of adenylate cyclase and allows cholera toxin to irreversibly activate the cyclase. Though apparently a component of the adenylate cyclase complex, the physiological role of ARf is unknown. ARF is present in the plasma membrane of every eukaryotic tissue or cell type examined. Attempts to isolate ARF deficient cell lines been unsuccessful in a number of laboratories. ARF has the same molecular mass and shares the GTP-binding characteristics of the 21 kDa ras oncogene. Ras proteins have been implicated as regulators of adenylate cyclase in yeast. The role of ras in mammalian cells is unknown.

Objectives

The overall objectives of this project include the elucidation of each component of the ARF pathway and determination of the role, if any, of ARF in transformation or oncogenesis. We will utilize many of the powerful genetic techniques to analyze the yeast ARF genes and use both DNA and antibody probes to address these questions. Thus, the cloning and sequencing of two ARF genes will be the starting place.

Methods Employed

We are currently finishing up work on the cloning and sequencing of two yeast ARF genes, ARF1 and ARF2, as well as human ARF and Xenopus ARF. Human ARF cDNA will be used to screen tissue obtained from human tumors for the levels of ARF mRNA and DNA in blots. The yeast genes will be used in disruption experiments as well as being used in inducible yeast expression vector, pBM272. Techniques for manipulation of yeast cells and genes can be found in the Cold Spring Harbor Manual for its Yeast Course. Synthetic peptides will be injected into rabbits for the production of mono-specific antibodies. Injection of ARF protein and antibodies into Xenopus oocytes will be performed in collaboration with Dr. Hsiang-fu Kung at FCRF, Frederick, MD.

Major Findings

Two ARF genes, ARF1 and ARF2 have been cloned from a genomic yeast library. Both genes have been mapped to chromosome IV. The amino acid sequences are more than 96% identical. Disruption of ARF1 results in three phenotypic changes: slow growth at 30°C, cold sensitivity, and supersensitivity to growth in the presence of fluoride ion. Suppressors of fluoride supersensitivity have been obtained and are currently being classed. There are at least three independent genes capable of suppressing the ARF1 disruption. Further characterization of these genes, both genetic and sequencing, is planned. Completion of the sequencing of ARF2 may yield further information on the relationship between these two genes. Disruption of ARF2 either alone or in conjunction with ARF1 will be of great interest to these studies and is currently in progress.

A human ARF gene has been cloned and partially sequenced. The coding region appears to be 100% identical to the bovine gene. Thus, the mono-specific bovine antibodies already in hand will be excellent probes for the human protein as well. We are currently trying to determine if man has one or more ARF genes.

We have successfully put the bovine and yeast ARF1 genes into bacterial and yeast inducible expression vectors, respectively. Expression of bovine ARF in bacteria results in about 20% of cell protein being ARF and purification of this expressed protein is underway. The expressed protein appears similar to purified bovine brain ARF with respect to nucleotide binding.

Proposed Course

1) Finish sequencing the ARF2 gene. Map both ARF genes on chromosome IV.
2) Disrupt ARF2 and determine phenotype, compare to arf1. Also do the double disruption, $arf1^{-}arf2^{-}$. Prediction is that double disruption will be lethal.
3) Immunolocalization of ARF proteins in fixed yeast cells. If possible prepare better yeast ARF antibodies as well as antibodies specific to each of ARF1 and ARF2.
4) Put human ARF into inducible yeast expression vector. Put into WT, arf1, arf2, arf1arf2 cells. Does human ARF complement arf1 or arf2?
5) Clone and sequence the Xenopus ARF gene. Delete Xenopus ARF gene and determine phenotype. Microinject ARF proteins or antibodies into Xenopus oocytes and monitor development. Also monitor cellular levels of cAMP, phosphoinositides, both in control and hormone stimulated oocytes.

Publications

1. Zaremba T, Gierschik P, Pines M, Bray P, Carter A, Kahn R, Simons C, Vinitsky R, Goldsmith P, Spiegel A. Immunochemical Studies of the 36-kDa β Subunit of Guanine Nucleotide-Binding Proteins: Identification of a Major Epitope. Mol. Pharm., 33:257-264, 1988.

2. Kahn RA. Regulators of signal transduction: families of GTP-binding proteins. In: Glazer R, ed. Developments in Cancer Chemotherapy. Boca Raton: CRC Press, 1988 in press.

3. Kahn RA. The ADP-ribosylation Factor of Adenylate Cyclase: a 21 kDa GTP-binding Protein. In: Birnbaumer L, Iyengar R, eds. G-Proteins, Academic Press, 1988, in press.

4. Sewell J, Kahn RA. Sequences of the Bovine and Yeast ADP-ribosylation Factor and Comparison to other GTP-binding Proteins, Proc Natl Acad Sci, USA 1988 in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06190-02 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on ona line between the borders.)*

Protein Phosphorylation in Multidrug-Resistant Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert I. Glazer	Supervisory Pharmacologist	LBC NCI
Others:	Angelo Aquino	Visiting Fellow	LBC NCI
	Marian Johnson Thompson	IPA	LBC NCI
	Marian Knode	Biologist	LBC NCI
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SECTION

INSTITUTE AND LOCATION

NCI, NIH, BETHESDA, MARYLAND 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
4.50	3.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unraduced type Do not exceed the spaca provided.)*

The purpose of this project is to characterize protein phosphorylation and the associated Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) activities in multidrug resistant (mdr) cells. The overexpression of PKC is closely associated with the mdr phenotype in both leukemic and breast carcinoma cell lines. Since PKC is a family of enzymes with strikingly different responses to proteolysis and phorbol ester activation, one goal of this proposal will be to determine the relative abundance of the three major isoforms of PKC in mdr cells in comparison to sensitive cells using chromatographic and protein blotting techniques. A second aim of this project is to assess the phosphorylation of the mdr-associated P-glycoprotein (Pgp) and to determine whether modulation of the phosphorylation of this protein can affect the degree of drug resistance. This will be accomplished by determining: 1) whether specific inhibitors of PKC such as K252b and calphostin C or activators of PKC such as TPA and bryostatin can block or stimulate, respectively, the phosphorylation of Pgp in vivo and in vitro and 2) whether transfection with PKC-τ or -α of wild type, multidrug resistant or wild type cells previously transfected with the mdr-1 gene can alter the degree of sensitivity or resistance of these cells.

Objectives.

The purpose of this project is to characterize protein phosphorylation and the associated Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) activities in multidrug resistant (mdr) cells. The overexpression of PKC is closely associated with the mdr phenotype in both leukemic and breast carcinoma cell lines. Since PKC is a family of enzymes with strikingly different responses to proteolysis and phorbol ester activation, one goal of this proposal will be to determine the relative abundance of the three major isoforms of PKC in mdr cells in comparison to sensitive cells using chromatographic and protein blotting techniques. A second aim of this project is to assess the phosphorylation of the mdr-associated P-glycoprotein (Pgp) and to determine whether modulation of the phosphorylation of this protein can affect the degree of drug resistance. This will be accomplished by determining: 1) whether specific inhibitors of PKC such as K252b and calphostin C or activators of PKC such as TPA and bryostatin can block or stimulate, respectively, the phosphorylation of Pgp in vivo and in vitro and 2) whether transfection with PKC-τ or -α of wild type, multidrug resistant or wild type cells previously transfected with the mdr-1 gene can alter the degree of sensitivity or resistance of these cells.

Significance.

The cell biology of multidrug resistance has centered on the role of cell membrane-associated proteins termed collectively, Pgp. Previous studies on the development of drug resistance have focused on alterations in the properties of this cell membrane component. Amplified membrane glycoproteins have been identified in several cell lines exhibiting mdr. Recent transfection experiments with the genomic or complementary DNA encoding an mdr-associated Pgp have established that this glycoprotein confers multidrug resistance to sensitive cells. In contrast, studies of the posttranslational modification, eg. phosphorylation, of Pgp have been limited. A 100-fold Adriamycin-resistant cell line of promyelocytic leukemia cell line HL-60 contained two membrane glycoproteins of 110 and 160 kDa which were not present in the parental cell line. This cell line also exhibited cross resistance to vinblastine and actinomycin D as well as enhanced drug efflux, characteristics typical of mdr cells. Similar phenotypic changes have been noted in other HL-60 cell lines that were 10-fold and 80-fold resistant to Adriamycin and which contained membrane proteins of 120-250 kDa that were not only absent in wild type cells but were also phosphorylated *in vitro* in the resistant cell line. However, it has not been demonstrated whether the latter cell lines display true multidrug resistance. Similar studies in Chinese hamster lung cell cells resistant to Adriamycin revealed *in vitro* phosphorylated proteins of 180 and 220 kDa. A recent report described the phosphorylation *in vivo* of the 170-180 kDa glycoprotein in K-562 cells that were 150-fold resistant to Adriamycin. In the latter study, drugs such as verapamil and trifluoperazine which inhibit drug efflux and restore drug sensitivity to mdr cells increased phosphorylation of the membrane glycoproteins above its basal level. This implies that phosphorylation of transport or drug-binding proteins may regulate the efflux of drugs and other xenobiotics in mdr cells. However, the direct association

between phosphorylation of any normal cellular or mdr-associated protein and the function of that protein remains to be established.

Major findings.

In vitro phosphorylation assays carried out with extracts of HL-60/ADR cells showed the Ca^{2+}-and phospholipid-independent phosphorylation of a 130 kDa protein which was not present in parental HL-60 cells except after treatment with TPA. The 130 kDa protein was antigenically related to vinculin. This phenomenon did not occur in the parental cell line. Since HL-60/ADR cells exhibit the ability to phosphorylate vinculin in vitro in the same manner as TPA-treated wild type cells, and since PKC is believed to be a receptor for phorbol esters, we examined the role of this enzyme in the ability of HL-60/ADR cells to phosphorylate vinculin. DEAE-Sepharose chromatography of cell extracts revealed that HL-60/ADR cells contained 2-fold more PKC than the parental cell line. PKC activity was found only in the cytosol of wild type HL-60 cells, whereas 85% of the PKC activity was cytosolic and 15% was membrane-bound in HL-60/ADR cells. After treatment for two days with 10 nM TPA, PKC activity was reduced 80-90% in both cell lines regardless of its intracellular distribution. Coincident with TPA treatment of HL-60/ADR cells was a reduction in Ca^{2+}- and phospholipid-independent phosphorylation in vitro of vinculin whereas, TPA-treated wild type cells exhibited elevated levels of this phosphoprotein. The phosphorylation of vinculin in TPA-treated HL-60 or untreated HL-60/ADR cells was blocked in vitro by goat polyclonal antibodies to PKC or rabbit polyclonal antibodies to vinculin. These results suggest that it is not only the absolute level of PKC but also the proteolytic activation of PKC to P_{KM} which is associated with the utilization of vinculin as an endogenous substrate.

Quantitation of PKC and PKM by Western blotting using polyclonal antibodies to the consensus sequence AYQPYGKSVD in the catalytic domain of PKC has demonstrated that one of the two isoforms of PKC present in HL-60 cells is more abundant in HL-60/ADR cells and that substantially more PKM is generated in these cells. These studies have been expanded to an investigation of the α, α, β, τ, δ and ϵ isoforms of PKC. We have used the isoform-specific sequences in the "hinge" region just distal to the proteolytic cleavage site to prepare antipeptide polyclonal antibodies in rabbits. The sequences are shown in Figure 1. It should be noted that the variable region sequences occur C-terminal to the tryptic cleavage site present in PKC-α and PKC-β, and thus can detect the catalytic region or PKM.

The isoform pattern of protein kinase C (PKC) was examined in wild-type and Adriamycin-resistant (HL-60/ADR) HL-60 leukemia cells. Analyses were carried out by immunoblotting with rabbit polyclonal antibodies against isoform-specific peptides contained in the variable (V3) region of PKC-α, β and τ. HL-60 cells contained mainly PKC-β and τ and low levels of PKC-α. HL-60/ADR cells contained an equivalent level of PKC-β, more PKC-τ and considerably greater levels of PKC-α. Treatment of HL-60 cells with the phorbol ester, TPA, for 48 h increased the amount of PKC-α and -β' but did not affect the amount of PKC-※. HL-60/ADR cells treated with TPA exhibited a marked reduction in PKC-α and

V3 VARIABLE REGION

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PKCα 300  ELRQKFEKAKLGPAGNKVISPSEDRRQ------PSNN--LDRVK-----L
PKCβ 300  ELRQKFERAKIGPGPKTPEEKTINTISK-----FDNNGNRDRMK-----L
PKCτ 298  LL-QKFE-ACNYPLELYERVRIGPSSSPIPSPSPSPTDSKRCFFGASPGRL
PKCδ 309  GIYQGFEKKTAVSGNDIPDNNGTYGKIWEGSNRCRLENFTFQKVLGKGSFG
PKCε 318  QRRKKLAAGAESPQPASGNSPSEDDRSKSAPTSPCDQELKELENNIRKALS

consensus L-QKFE-A---P----------------------------------L
for α,β,

trypsin       KFEKAK
cleavage site
for PKC-α or -β
```

Figure 1. The V3 variable region sequences in the isoforms of protein kinase C. Also shown is the epitope recognition site for tryptic cleavage of PKC-α or -β.

little or no change in PKC-β and -τ, however, TPA induced a new 95 kDa species of PKC-τ. These results indicate that the isoform pattern of PKC differs between sensitive and ADR-resistance HL-60 cells. In addition, the different effects of TPA on PKC-α, -β and -τ, as well as the appearance of a higher molecular weight form of PKC-τ only in resistant cells indicates that the regulation of this family if isoenzymes is altered in drug-resistance cells.

We are presently investigating three other aspects of this proposal. First, we have developed a high resolution two-dimensional electrophoresis system for characterizing the phosphorylation of endogenous substrates in the mdr cell lines. Our findings thus far, indicate that vinculin is not phosphorylated in HL-60/ADR cells by metabolic labeling with $H_3{}^{32}PO_4$, in contrast to our previous results in vitro with cell extracts. In addition, these cells do not appear to contain Pgp as assayed by Western blotting with monoclonal antibody C219 (Centocor, Inc.). However, these cells do contain a large amount of PKM related to PKC-α and -β, as well as a high amount of a DNA-binding protein which may be phosphorylated by PKC or PKM. We are presently investigating the latter possibility with respect to both the role of phosphorylation and transcriptional activation in the type of resistance expressed in this HL-60 cell line. It should be noted that there were no differences in the level PKM or DNA-binding proteins in the mdr cell line MCF-7/ADR obtained from Dr. Ken Cowan, NCI which has the same degree of resistance (200-fold) to Adriamycin as HL-60/ADR cells, but contains Pgp. Therefore, we may have stumbled on a mechanistic difference between the type of resistance related to Pgp and the phenotype which does not express this protein. We are also exploring the possibility that the resistance exhibited by HL-60/ADR cells is related to the phosphorylation (hence activation) of topoisomerase II with Dr. Ives Pommier, NCI. Second, we are collaborating with Dr. Marian Myers, Sloan-Kettering Institute to study the role of Pgp phosphorylation in her Chinese hamster lung cell line DC-3F/AD-X which is 10,000-fold resistant to actinomycin-D and 340-

fold resistant to Adriamycin, and which expresses an abundance of Pgp. This cell line should be a good model for determining the influence of phosphorylation of Pgp on resistance. Third, we have cloned both the sense and antisense cDNA for PKC-τ into an expression vector for determining the influence of PKC expression or its absense of expression in wild type and resistant MCF-7 cells. We plan to determine: a) if expression of the antisense PKC-τ reverses resistance in HL-60/ADR cells, b) if expression of antisense PKC-τ alters resistance and Pgp phosphorylation in MCF-7/ADR and DC-3F/ADX cells, and c) if expression of PKC-τ in wild type cells confers resistance to these cells. We also plan to compare the expression of the antisense cDNA encoding PKC-τ with antisense thiooligonucleotides encoding the initiation sequence for this isoform for their effects on resistance in the various cell lines.

Experimental Design and Methods.

Human promyelocytic cell line HL-60 was obtained from the American Type Culture Collection. HL-60/ADR cells displaying multidrug resistance was provided by Dr. Steven Grant, Medical College of Virginia. Human breast carcinoma cell line MCF-7 rendered resistant to Adriamycin or vinblastine and wild type MCF-7 cells transfected with the mdr-1 gene were provided by Dr. Kenneth H. Cowan, Medicine Branch, NCI. DC-3F/ADX and DC-3F cell lines were provided by Dr. Marian Myers, Sloan Kettering Institute. The conditions for cell culture have been described previously.

PKC assays and the immunoblotting and detection of PKC, PKM and vinculin were performed as described previously. The concensus peptide AYQPYGKSVD in the catalytic domain of PKC α, β and τ were synthesized using a Beckman amino acid synthesizer by Dr. Chien Niu, NCI. The unique sequences, AGNKVISPSEDRRQ, GPKTPEEKTTNTIS, and LELYERVRTGPSSS, in the variable V3 region of PKC α, β and τ respectively, were synthesized by Drs. Chien Niu and James Omichinski, NCI. Polyclonal antibodies will be prepared by coupling each peptide to keyhole limpet hemocyanin with glutaraldehyde and immunizing rabbits using the adjuvant MPL+TDM+CWS (Ribi Immunochem Research).

Cell extracts were prepared in a Triton X-100 buffer containing the protease inhibitors leupeptin, aprotinin, pepstatin and phenylmethylsulfonyl fluoride (leupeptin is omitted when Ca^{2+}-dependent protease effects are assessed). Phosphorylation assays were performed in the presence and absence of Ca^{2+} and phosphatidylserine to quantitate PKC-dependent phosphorylation. The phosphorylated proteins were separated by one- or two-dimensional polyacrylamide gel electrophoresis. Western blotting of the gels onto nitrocellulose were carried out by the Towbin procedure using the Proto Blot (Promega Biotec) procedure for the detection of the antigen. For analysis of phosphorylation in vivo, cells will be incubated with $H_3{}^{32}PO_4$ for 4 hr in medium containing 0.1X phosphate.

Publications.

Aquino A, Hartman KD, Knode MC, Grant S, Huang KP, Niu C-H, Glazer RI, The role of protein kinase C in the phosphorylation of vinculin in Adriamycin-resistant HL-60 leukemia cells, Cancer Res 1988; 48:384-388.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06193-01 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tryptophan and its Antimetabolites in Control of Protein Synthesis and in Chemotherapy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Marco Rabinovitz Research Chemist LBC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.05	1	.05

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Analysis of the characteristics of tryptophan antimetabolites on cell culture of L1210 murine leukemia cells revealed a unique feature of 4-fluorotryptophan not shared by its 5- and 6- fluoro- positional isomers. Only the 4- isomer was cytotoxic in culture at concentrations equal to that of its natural metabolite, tryptophan, a characteristic which should make it effective in vivo. Cytotoxicity was manifested by growth inhibition, cell swelling and lysis. In vivo studies employing continuous release pellets implanted SC indicated its limit for host toxicity. Reports that the cytotoxicity of interferon-τ is due to induction of indoleamine-2, 3-oxygenase which degrades cellular tryptophan and acts by causing a tryptophan deficiency has prompted initial studies on combination chemotherapy with the biological response modifier and antimetabolite.

PHS 6040 (Rev. 1/84) GPO 914-918

Objectives.

It is the purpose of this project to develop within one unit, the broad objectives specified for the Laboratory of Biological Chemistry, including evaluation of possible targets involving cell signaling for chemotherapeutic intervention, through to the development of "appropriate in vivo systems to evaluate the chemotherapeutic effectiveness of agents shown to be active in simpler in vitro model systems". The amino acid tryptophan has been chosen as a key nutrient for investigation involving characterization of the "signal" to disaggregate polyribosomes which is exhibited by cells during amino acid deficiency and we have initiated development of a therapeutic regimen with one of its analogs unique in its efficacy in substituting for tryptophan in protein synthesis.

Major Findings.

A. In Vitro Studies.

An investigation of several antagonists to the essential amino acids indicated that many cause cell damage to L1210 murine leukemia cells when incubated in culture medium deficient in the natural amino acid but only one, 4-fluoro-tryptophan was effective in the presence of an equimolar concentration of its natural metabolite, tryptophan. Activity, as seen by growth inhibition, cell swelling and lysis was observed also with the 5-fluoro- and 6-fluoro-positional isomers but only in tryptophan deficient medium. An equimolar concentration of tryptophan blocked their activity. A survey of the literature indicated the surprising observation that 4-fluorotryptophan is preferred over the natural tryptophan for growth of certain bacterial mutants. This indicates that its `cytotoxicity' is of a character other than that of the usual `cytotoxic agents' and makes it an appealing prospective candidate for cancer chemotherapy.

B. In Vivo Studies.

We have developed a palatable tryptophan deficient ration based on both an amino acid mixture and the corn protein, zein. Adult mice have been maintained on this ration for over a month. Such deficient rations have been reported as necessary for eliciting responses to antagonists in vivo, since the availability of the natural metabolite may be too high under normal nutritional conditions.

A pellet, designed to deliver 4-fluorotryptophan continuously for a period of one week when implanted subcutaneously, was prepared by the company, Innovative Research of America. When 50 or 25 mg pellets were implanted in the back of the neck, mice died in 2 days. Placebos were not toxic. Since tryptophan is also toxic to mammals in high doses because of its metabolism to serotonin, current approaches are being evaluated to block such metabolism with the use of hydroxylation and decarboxylation inhibitors.

Proposed Course.

A. 4-Fluorotryptophan Studies. Much of the proposed course for development of 4-fluorotryptophan has been discussed within the major findings section. It is also proposed to prepare the L form of this of this amino acid analog by combining 4-fluoroindole and serine through the agency of tryptophan synthetase. Dr. Edith W. Miles of NIDDKD has kindly consented to supply the bacterial enzyme. This approach should also permit a route for the preparation of the labeled analog by using radioactive L-serine. Uptake and incorporation studies will be evaluated with this labeled compound. A large body of literature has indicated that interferon-τ (immune interferon) exhibits its cytotoxicity through inducing indoleamine 2,3-dioxygenase, which destroys tryptophan in tumor cells and induces a tryptophan deficiency. Since tryptophan overcomes the cytotoxicity of interferon-τ, 4-fluorotryptophan and this biological response modifier should act synergistically. This concept is being studied in vitro and will be evaluated in vivo.

B. Signal To Stop Initiation of Protein Synthesis Induced By Amino Acid Deficiency.

1) Use of cytoplasts for evaluation of the response of their ribosomal subunit and ribosome-polyribosome profile to conditions of tryptophan deprivation.

Cytoplasts from an Ehrlich ascites cells, fibroblasts and differentiated murine erythroleukemia cells will be prepared and evaluated for protein synthetic capacity and sensitivity to tryptophan deprivation alongside their normal nucleated cells of origin with the use of L-tryptophanol and α-methyltryptophan which block aminoacyl tRNA formation. Evidence that such macromolecular synthesis still occurs in cytoplasts has been documented by others. The cytoplasts and their normal nucleated cells of origin will then be lysed, their ribosome-polyribosome profile will be analyzed and the characteristics of the ribosomal subunits determined as we reported previously. Evaluation of these parameters could furnish definative evidence on the role of nucleus in amino acid control of peptide chain initiation.

The use of tryptophan and its antagonists in these studies should be especially propitious because 1) This amino acid has been shown to act at the nucleo-cytoplasmic membrane of liver cells to regulate RNP traffic. 2) Tryptophan is absent in a wide range of proteins, including all histones. Since histone H1 has been implicated in control of protein synthesis of diabetic muscle, the synthesis of this histone will not be inhibited because of the absence of one of its component amino acids. 3) The genetic code for tryptophan does not include any wobble for codons, thus minimizing complications of isoacceptor tRNAs.

2) The reticulocyte lysate as an assay system for cytoplasmic effectors of other cell types which mediate a block in peptide chain initiation upon antagonism to tryptophan activation.
Studies designed to determine the lesion associated with inhibition of

initiation have involved estimation of initiation factors or complexes in cells or their lysates after deprivation. Since these may involve changes resulting from a cascade of events during deprivation, a `pleotropic' effect, the use of a standard reticulocyte assay system for evaluation is proposed. Cytoplasmic fractions of nucleated cells, e.g. Ehrlich ascites cells, which had been incubated under deficiency conditions, will be added to the standard reticulocyte lysate to determine their possible role in polyribosome disaggregation. Since we have shown that the ribosome-polyribosome profile of reticulocytes remains fixed during deficiency, disaggregation should be due only to the added fraction and not any secondary event. In this way preliminary studies on the mechanism of action of our hemin controlled translational repressor (HCR) were carried out. If an active fraction is found, it will be isolated by gel filtration according to molecular size prior to further purification procedures.

Publications and Patents.

Rabinovitz M, The structure of flavone-8-acetic acid, a chemotherapeutic agent, and its application to drug design, J Enzyme Inhibition 1988; 2:151-152.

Rabinovitz M, US Patent 4,755,515: Chemotherapeutic 1-(2-chloroethyl)-4-(3-chloropropyl)-piperazine,dihydrochloride, July 5, 1988.

Rabinovitz M, Fisher JM, Evidence for a copper, S-(methylthio)-L-homocysteine complex as a glutamine antagonist of cytidine triphosphate synthesis in L1210 murine leukemia cells, Mol Pharmacol 1988; 34: 401-406.

Rabinovitz M, Emerging evidence for control of monovalent cation homeostasis as a critical target in alkylating agent resistance, in Resistance to Antineoplastic Drugs, Kessel D, ed. CRC Press 1989; 257-266.

Rabinovitz M, Flavone-8-acetic acid and the natural flavonoids, J Natl Cancer Inst 1989; 81:962.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06196-01 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of HMG CoA Reductase Activity by Cell Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S Friedman Supervisory Pharmacologist LBC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Previous studies from this laboratory have shown that cell-cell interactions mediate the negative feedback regulation of growth rate in mammalian cell populations and the activities of several key regulatory enzymes in sterol metabolism including HMG CoA reductase. The purpose of this project is to determine the mechanism(s) of regulation of isoprenoid metabolism by cell interactions in glial tumor cells. The first objective is to examine the basis for the density-dependent decline in HMG CoA reductase levels; the second is to investigate the basis for the apparent coregulation of HMG CoA reductase activity and growth rate by cell density; and the third is to study the consequences of density regulation on sterol biosynthesis and other mevalonate-utilizing pathways.

PHS 6040 (Rev 1/84) GPO 914-918

Previous studies from this laboratory have shown that cell-cell interactions mediate the negative feedback regulation of growth rate in mammalian cell populations. This constitutes a mechanism for controlling both the momentary tissue size and the extent of compensatory growth of a tissue following cell loss. An understanding of the molecular basis for tissue sizing may uncover new chemotherapeutic targets in solid tumors and lead to a more effective management of compensatory tumor growth.

Cellular growth requires the coordination of multiple biosynthetic and assembly processes. A useful first step in understanding how tissue sizers operate may be to study biosynthetic processes that are regulated by cell interactions and to determine the cellular mechanisms for translating information about population size into qualitative and quantitative changes in biosynthetic activity. We are currently using this approach to study the regulation of isoprenoid metabolism in glial tumor cells. Isoprenoids are required for multiple cell functions including membrane biosynthesis, glycosylation, nuclear lamin assembly, mitochondrial respiration and cell cycle progression; it is not surprising therefore that a number of plant and marine natural products with antitumor activity are also biosynthetic derivatives of this pathway.

We have initiated this study by focusing on HMG CoA reductase, an enzyme that is known to play a major regulatory role in the biosynthesis of sterols and other isoprenoids. Previous studies in our laboratory established that the activity of this enzyme and ACAT, another key enzyme involved in the regulation of intracellular sterol levels, can be controlled by cell interactions. In these experiments, cell interactions were varied by manipulating cell culture density. Density regulation of HMG CoA reductase occurred in a number of cell types, including MDCK renal adenocarcinomas, rat C6 gliomas and normal human fibroblasts. Detailed studies were carried out primarily with C6 gliomas which showed that: enzyme activity varied inversely with cell density over a broad range; the effect was not mediated by nutritional depletion, by diffusible- or matrix-bound molecules, or by LDL receptors; enzyme activity decreased irreversibly with biphasic kinetics when cells were abruptly shifted to higher density; and density regulation of the enzyme occurred in cells that were blocked in the synthesis of oxysterols, which have been implicated in sterol-mediated losses of enzyme activity.

Objectives:

The goals of this project are: (1) to determine the molecular basis for density dependent regulation of HMG CoA reductase levels; (2) to investigate the basis for the apparent coregulation of HMG CoA reductase activity and growth rate by cell density: and (3) to study the consequences of density regulation on sterol biosynthesis and other mevalonate-utilizing pathways.

Major Findings:

1. Density regulation of HMG CoA reductase involves changes in enzyme levels. Procedures were developed to quantitate HMG CoA reductase on immunoblots. When

enzymatically active cell lysates were fractionated by SDS-PAGE, transblotted to nitrocellulose, and reacted with rabbit anti-rat HMG CoA reductase or nonimmune serum, followed by GAR-alkaline phosphatase, 2 major bands with apparent MWs of 68 and 64 Kd and a minor band of approximately 34 Kd were specifically stained by immune serum. The level of the 64 kd band decreased in parallel with enzyme activity as cell density increased. These bands may be nonspecific proteolysis fragments of the native enzyme which, however, has never been detected in significant amounts in these experiments, even when cells are ruptured in the absence of detergents and in the presence of as many as 11 different antiproteases and EDTA. The cleavage pattern differs from that produced by lysosomally-contaminated membrane fractions where as many as 10 antigenically reactive bands ranging from 67 Kd-17 Kd are present. The most recent results, obtained using nitrogen cavitation to disrupt cells in a sucrose-tris-magnesium chloride-potassium chloride buffer containing multiple antiproteases, produced minor bands at 130 and 67 Kd and a major band at 53 Kd, which was also the predominant size of newly synthesized enzyme detected in experiments using 35S-methionine pulse-labeling with immunoprecipitation. Further attempts to stabilize the enzyme will be made by breaking cells under conditions that stabilize microtubules and/or by using digitonin-permeabilized cells layers that preserve the integrity of both the ER and microtubules.

(a). Measurement of synthesis and degradation rates and follow-up experiments. As soon as procedures are developed for minimizing spontaneous proteolysis, rates of synthesis and degradation will be measured as a function of cell density. During the past several months, I optimized procedures for measuring synthesis rates and determined that the rate of enzyme synthesis decreased as cell density increased. A comparison of changes in enzyme levels and enzyme synthesis suggested however that density regulation might operate primarily through changes in degradation rate. If this proves to be the case, the mechanism by which enzyme degradation is regulated will be addressed as follows.

Density-jump experiments will be used to determine whether irreversible inactivation is an obligatory stage in the enzyme degradation process. Previous studies using this protocol indicated that enzyme activity declines with biphasic kinetics which suggests the possibility of a two-step mechanism. Studies on permeabilized cell layers also suggests that degradation may be preceded by irreversible inactivation of the enzyme. An analysis of the enzyme's size distribution and activity at multiple times after density jumping should confirm or disprove this hypothesis. If inactivation precedes degradation, further experiments will test the possible involvement of covalent modification of the enzyme or its complexation with a tight-binding inhibitor in this process.

2. Changes in mRNA levels occur only at densities approaching confluency.

Total RNA was extracted from cells cultured at different densities and fractionated on Northern gels. Analysis by Northern blot hybridization to a full length HMG CoA reductase cDNA probe revealed two hybridizable RNA species at 4.4 kb and 2.4 kb. The band intensities, which were linearly proportional

to radioactivity, were quantitated by densitometry. Slight differences in the amount of RNA applied to each lane were corrected by normalizing results to ribosomal RNA detected by filter rehybridization to a ribosomal DNA probe. The results of these experiments showed that mRNA levels remained relatively constant until cells were approximately 80% confluent at which time the 2.4 kb species precipitously declined, whereas the 4.4 kb species declined by only 30%.

Although multiple classes of mRNA distinguished by differences in 5' initiation sites have been reported in CHO cells, it is uncertain whether the 4.4 kb and 2.4 kb bands from gliomas represents different mRNAs or, alternatively, whether the two species were artifactually generated by entrapment in comigrating ribosomal RNA species. To clarify this, the experiment will be repeated with poly A+ RNA. Nuclear and cytoplasmic fractions will also be analyzed to determine whether the two bands represent primary and/or processed transcripts.

The observation that enzyme levels decline continuously from sparse to confluent densities while RNA levels only begin to change when cells are at relatively high density suggests the existence of two modes of density regulation of HMG CoA reductase which may correspond to differences in cell cycle controls over growth rate that we reported previously for this cell line. In those studies, we found that over a broad range of subconfluent densities, density regulation occurred by means of changes in the relative duration of S phase. As cultures approached and passed confluency however, changes in doubling time increasingly reflected the pile up of cells in G1 phase. In the near future, we hope to be able to resume these experiments and to determine whether the transcription or stability of HMG CoA reductase mRNA is cell-cycle regulated.

3. Studies on the role of microtubules as a coordinating mechanism for density-mediated regulation of HMG CoA reductase activity.

The recently discovered role of interphase microtubules in the morphogenesis and stability of endoplasmic reticulum suggests a possible mechanism for coordinating the rates of several growth-associated biosynthetic processes with changes in population size. By controlling the turnover rates of selected regulatory enzymes in the endoplasmic reticulum, changes in microtubule dynamics produced by growth-induced changes in cell contact interactions could conceivably alter the rates of growth-associated biosynthetic pathways, for example, those required for membrane biogenesis. This hypothesis might explain the observation that sterol and lipid synthesis, both of which are controlled primarily by enzymes in the endoplasmic reticulum are coregulated by density.

This hypothesis predicts features of HMG CoA regulation that are not intuitively obvious.

The first is that steady-state changes in the level of the enzyme (which has a short half-life) can be produced by changes in microtubule polymerization and depolymerization. My previous experiments confirm those reported by another laboratory that a reversible dose-dependent decrease in HMG CoA reductase

activity is produced by treating C6 gliomas with antimicrotubular drugs. Using fluorescent probes for microtubules and endoplasmic reticulum, it should be possible to determine whether changes in the level of the enzyme are closely correlated with changes in the organization of these cellular organelles.

Secondly, abrupt changes in the half-life of the enzyme should occur at mitosis when the cytoplasmic microtubular network and associated endoplasmic reticulum are disassembled, and again in early G1, when these structures reform.

Thirdly, the accelerated degradation of HMG CoA reductase mediated by a nonsterol metabolite of mevalonic acid may depend on changes in microtubular dynamics and/or microtubule-ER interactions induced by covalent modification of microtubule-associated proteins. It is known that mevalonate causes cell rounding at concentrations in excess of those required for negative feedback regulation of the enzyme and that tubulin becomes labeled by incubation of cells with radioactive mevalonic acid.
Experiments will be carried out to test the plausibility of this hypothesis.

Publications:

Matheson DS, Green B, Friedman SJ, Hoar D, Studies on the mechanism of activation of human natural killer function by interferon and inhibitors of thymidylate synthesis, Cellular Immunology 1988; 111:118-125.

Skehan P, Friedman SJ, Tissue sizing-a community control of neoplastic growth, In Paukovits WR (ed), Growth Regulation and Carcinogenesis, CRC press 1989; in press.

Skehan P, Friedman SJ, Cancer- a disease of tissue neogenesis?, In Paukovits WR (ed), Growth Regulation and Carcinogenesis, CRC press 1989; In press.

Friedman SJ, Skehan P, The Etiology of Human Neoplasms, In Dulbecco R (ed), Encyclopedia of Human Biology; In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07156-06 LBC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Differentiation of Human Leukemia Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Theodore R. Breitman	Chemist	LBC, NCI
Others:	Noriko Takahashi	Visiting Fellow	LBC, NCI
	Yuya Abe	Visiting Fellow	LBC, NCI
	He Ruyi	Visiting Fellow	LBC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent clinical results are supportive that induction of differentiation is an alternative approach for the treatment of some malignancies. Our studies are involved with gaining additional knowledge on the process of terminal differentiation, the mechanism of action of inducers, and finding clinically useful combinations of inducers. Studies were conducted to: a) measure changes in the levels of the c-myc oncogene protein (c-Myc) during differentiation and to correlate changes in the levels of c-Myc with other changes associated with differentiation; b) study the metabolism of retinoic acid (RA), a potent inducer of differentiation; and c) conduct an extensive analysis of the nature of the interaction of combinations of inducers of differentiation. The human myeloid leukemia cell line, HL60, has been a useful model system for studying terminal differentiation. A method was developed to fix and permeabilize these cells so that intracellular antigens could be detected with specific antibodies and measured with a fluorescence activated cell sorter (FACS). We examined the levels of c-Myc during RA- and dimethylsulfoxide-induced differentiation and found that both the rate and the extent of decreases in c-Myc during differentiation are much less than the decreases in c-myc mRNA levels under similar conditions. Although many biological effects of RA have been described, the mechanism for these actions is unknown. We have now discovered that in many cell lines, a covalent bond is formed between RA and protein. In HL60 there is only one major retinoylated protein species and it is localized in the nucleus. Based on sensitivity to hydrolysis with either hydroxylamine or methanolic-KOH, the RA moiety is probably linked to protein via a thio-ester bond. Retinoylation occurs at very low concentrations of RA. Evidence supporting a functional role for this low-level retinoylation is that combinations of RA and either dimethylsulfoxide, hexamethylene bisacetamide, or sodium butyrate synergistically induce differntiation of HL60 cells.

Objectives.

This project seeks an understanding of the process of terminal differentiation of human myeloid cells. It is now generally agreed that some leukemias, as well as other malignancies, are diseases resulting from a block in terminal differentiation. This view suggests that viable treatment may be possible with agents that induce differentiation. To aid in this search, studies are conducted to: a) better understand the mechanism(s) of terminal differentiation; b) study the metabolism of known inducers of differentiation, e.g., retinoic acid (RA); c) screen known and newly synthesized compounds for their differentiation inducing activity either alone or in combination with differentiation-inducers.

Recent studies have been elucidating the mechanism of cell proliferation promoted by oncogenes and the relationship between oncogene expression and differentiation. Several oncogene products are related to growth factors or their receptors. Most studies on oncogene expression are based on measurements of transcriptional changes in a large population of cells. There have been relatively few studies on the translational products. Furthermore, there are relatively few studies in which oncogene proteins have been measured in individual cells as opposed to measurements on the population. Most of these later studies have been conducted with immunofluorescence microcopy. While this technique is primarily qualitative, important information has been obtained on both the percentage of cells positive for an antigen and localization of the antigen in the cell. However, it is difficult with immunofluorescent microscopy to estimate relative levels of specific antigenic proteins in individual cells and even more difficult (or at least laborious) to determine what percentage of a small population may be positive or negative. Alternative techniques to measure oncogene proteins include immunoprecipitation and Western blotting. While these techniques give fairly good quantitiation they measure changes in the population and therefore changes in a subpopulation of cells can be missed.

A fluorescence activated cell sorter (FACS) can measure the level of a specific molecule in individual cells of a large population. The ability to measure oncogene proteins with this instrument allows for a detailed examination of oncogene protein expression during growth and differentiation. In addition, the development of methods to measure intracellular antigens, a necessary prerequisite of FACS analysis of intracellular oncogene protein, should also find applicabilty in studies on non-oncogene intracellular antigens. Because other parameters such as cell size, granularity, and other antigens can also be measured on the same cells correlations of changes in oncogene protein expression with other parameters can be made. In addition, cells of interest can be isolated for further analysis.

Although the actions of RA have been investigated by many, the mechanism for these actions is unknown. RA supports growth in animals and maintains epithelial tissues and bone, but does not function in vision and mammalian reproduction. The importance of RA is underscored by evidence that it is a potent inducer of differentiation of some cell types and that it has utility in

the treatment of patients with various malignancies. In vivo and in some cell types there is an isomerization equilibrium between all-trans- and 13-cis-RA and also a conversion of the water-insoluble RA to the water-soluble retinoyl glucuronide (1-O-retinoyl-β-D-glucopyranuronic acid). Both 13-cis-RA and retinoyl glucuronide are active in some systems in vitro. However, there is essentially no information on reactions involving RA that are directly correlated with the effects of RA. We have speculated that RA is activated in a CoA-SH mediated reaction to form retinoyl-CoA. This high energy intermediate could then react with susceptible groups on a macromolecule e.g., an hydroxy group, to form a low energy covalent ester bond. We now have evidence that RA is covalently linked to HL60 protein through either an oxygen-ester or a thio-ester bond. Thus, the amino acids serine, threonine, tyrosine, and cysteine may be sites for this acylation. If this acylation competes with or modulates other modifications (phosphorylation, methylation, palmitoylation) at the same or closely associated sites, it could lead to a better understanding of the mechanism of action of RA as well as throw new light on the function(s) of these other post-translational modifications. Furthermore, if retinoylation is essential for the response of a cell to RA, then a measurement of retinoylation could be the basis for a predictive test for the potential clinical utility of RA.

Methods Employed.

The principal methods employed involve measurement of differentiation of human leukemia cell lines in cell culture. Most studies are conducted with the HL60 human myeloblastoid cell line. Differentiation is assessed primarily by morphology, the ability of cells to reduce nitroblue tetrazolium to a formazan, and with other cell-type specific assays. Measurement of proto-oncogene expression is by immunological techniques using flow cytometry, Western blotting, and immunoprecipitation. Studies on retinoylation use primarily two-dimensional gel electrophoresis as an analytical tool.

Major Findings.

1. Employing FACS we have obtained data showing that c-Myc decreases during differentiation of HL60 at a much slower rate than expected from the c-myc mRNA level. Immunofluorescence microscopy and Western blotting confirms the relatively slow rate of decrease in c-Myc and the localization of c-Myc in the nucleus. c-Myc is lost from the cells after treatment with DNase but not after treatment with RNase. These results are consistent with the reported DNA-binding properties of c-Myc.

2. We now have evidence that a covalent bond is formed between RA and proteins in growing HL60 cells. The extent of the retinoylation of HL60 protein is dependent on both time and RA concentration. Radioactive RA is released from labeled HL60 cells in a trichloroacetic acid soluble form after digestion with proteinase K and hydrolysis with hydroxylamine under mild conditions. These results are consistent with retinoylation of protein with the formation of thio- or oxygen-ester bonds.

3. The finding that retinoylation occurs at physiologic concentrations of RA (10 nM) prompted an investigation of whether this retinoylation was associated with greater sensitivity to other inducers of differentiation. Studies with dimethylsulfoxide, hexamethylene bisacetamide, and sodium butyrate showed that each of these agents was markedly more active in the presence of RA. Analysis of this interaction, based on the median effect principle, showed that this combiniation is synergistic. These results could be useful in designing clinical protocols that have utility in the treatment of some leukemias.

Proposed Course

1. In the retinoylation subproject we will examine other cell lines for the presence of retinoylated proteins. Of more immediate interest is the identification of the retinoylated HL60 nuclear protein and the clarification of the relationship between the sensitivity of a cell type to RA and the extent of retinoylation.

2. Our finding that RA in combination with either dimethylsulfoxide, sodium butyrate, or hexamethylene bisacetamide synergistically induces differentiation of HL60 raises the question of whether these findings have clinical utility. We plan to extent these studies by examining the interrelationship of RA with other inducers (e.g., vitamin D3) and attempt of extend these findings to see if fresh cells from leukemia patients will also respond in vitro to these combinations.

Publications.

Breitman TR, Sherman MI, In vivo model systems for differentiation Therapy of leukemia and solid tumors, In Waxman S, Rossi GB, Takau F, eds. The status of Differentiation Therapy of Cancer, Raven Press NY 1988; 263-275.

Imaizumi M, Breitman TR, Changes in c-myc, c-fms, and N-ras proto-oncogene expression associated with retinoic acid- induced monocytic differentiation of human leukemia HL60/MRI cells, Cancer Res 1988; 48:6733-8.

Takahashi N, Breitman TR, Retinoic acid acylation (retinoylation) of a nuclear protein in the human acute myeloid leukemia cell line HL60, J Biol Chem 1989; 264:5159-63.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The Laboratory of Biochemical Pharmacology was established in January, 1986, by the division of the former Laboratory of Pharmacology and Experimental Therapeutics into two components which are concerned respectively with the design and synthesis of antitumor drugs (Laboratory of Medicinal Chemistry) and the mode of action of new antitumor drugs (Laboratory of Biochemical Pharmacology). The Laboratory studies new agents which have originated within the Developmental Therapeutics Program and also agents derived from extramural sources in whose preclinical development the Program is playing a major role. Over the past three years, the Laboratory has also participated actively in elucidation of the cellular and clinical pharmacology of compounds with anti-HIV activity, currently under development within the Program.

During the past year, the cellular pharmacology of 2′,3′-dideoxy-2′-fluoro-arabinosyladenine (fddA) has been investigated in cultured Molt-4 cells. Unlike 2′,3′-dideoxyadenosine (ddA), this fluorinated nucleoside is wholly resistant to decomposition by acid, and as a consequence, exhibits excellent oral bioavailability. fddA is also about 10 times more resistant to attack by adenosine deaminase than is ddA; its deamination product, fddI, is completely refractory to phosphorylytic decomposition by purine nucleotides or phosphorylase. The half-life of parent drug in RPMI medium (10% calf serum) is 4 hr, which is markedly longer than that of ddA ($t_{1/2}$: 15 min). The anabolism of fddA to the 5′-diphosphate and 5′-triphosphate is 22 and 5 times more energetic than the analogous processes measured with ddA. Viewed in concert, these results warrant consideration of fDDA as an oral therapy for AIDS.

In the past year, in collaboration with the Clinical Oncology Branch, we have also investigated the influence of granulocyte-macrophage colony stimulating factor (GM-CSF) on the replication of HIV-1 in cells of monocyte/macrophage (M/M) lineage, and its effect on the anti-HIV activity and metabolism of several 2′3′-dideoxynucleoside congeners of thymidine in these cells in vitro. We found that replication of HTLV-III_{Ba-L} (a monocytotropic strain of HIV-1) and HTLV-III_B (a lymphocytotropic strain) is markedly enhanced in M/M, but not in lymphocytes, exposed to GM-CSF in culture. Moreover, GM-CSF reduced the dose of HIV required to obtain productive infection in M/M. Even in the face of this increased infection, GM-CSF also enhanced the net anti-HIV activity of AZT and several related congeners: 2′,3′-dideoxythymidine (ddT), 2′3′-dideoxy-2′3′-didehydrothymidine (D4T) and 3′-azido-2′3′-dideoxyuridine (AZddU). Inhibition of viral replication in GM-CSF-exposed M/M was achieved with concentrations of AZT and related drugs which were 10-100 times lower than those inhibitory for HIV-1 in monocytes in the absence of GM-CSF. Other dideoxynucleosides not related to AZT showed unchanged or decreased anti-HIV activity in GM-CSF-exposed M/M. To investigate the possible biochemical basis for these effects, we evaluated the metabolism of several drugs in M/M exposed

to GM-CSF. We observed in these cells markedly increased levels of both parent and mono-, di-, and triphosphate anabolites of AZT and D4T compared to M/M not exposed to GM-CSF. By contrast, only limited increases of endogenous competing deoxynucleoside-5′-triphosphate pools were observed following GM-CSF exposure. Thus, the ratio of AZTTP/dTTP and D4TTP/dTTP is several fold higher in GM-CSF-exposed M/M, and this may account for the enhanced activity of such drugs in these cells.

Taken together, these findings suggest that GM-CSF increases HIV-1 replication in M/M, while at the same time enhancing the anti-HIV activity of AZT and related congeners in these cells. These results may have implications in exploring new therapeutic strategies in patients with severe HIV infection.

Studies continued on the biochemistry and pharmacology of tubulin, a protein critical for cell division, which is the target protein for many antineoplastic agents.

There were four major projects with antimitotic compounds in the last year. 1) The agent 2,4-dichlorobenzyl thiocyanate was found to interact wiht high specificity with β-tubulin, forming a covalent bond with cysteine 239, resulting in inactivation of tubulin for microtubule assembly. 2) The mechanism of action of the most promising agents derived from the South African tree Combretum caffrum, combretastatin A-4 and combretastatin A-2, were studied in detail. They were found to bind extremely rapidly to tubulin at the colchicine site and dissociate more slowly from the protein. 3) Dolastatin 10, a highly cytotoxic pentapeptide derived from the sea hare Dolabella auricularia, was demonstrated to act as a potent noncompetitive inhibitor of vincristine and vinblastine binding to tubulin and to eliminate exchange of guanine nucleotide on tubulin. Several stereoisomers of the natural product were equally active as inhibitors of tubulin polymerization, as was a tripeptide fragment of dolastatin 10. 4) Mechanisms of binding to tubulin of colchicine analogs more active than colchicine itself were extensively studied. Enhanced activity was associated with a greater association rate constant.

The Laboratory's interest in tubulin-nucleotide interactions was carried forward in three project areas. 1) A careful evaluation of reaction conditions on the effects of nonhydrolyzable GTP analogs on tubulin polymerization was performed. 2) Following reports in the literature that beryllium fluoride stabilized microtubules by acting as a phosphate analog, we have been evaluating beryllium fluoride in our reaction systems. 3) The GTP analog 2′,3′-dideoxyguanosine 5′-(α,β-methylenetriphosphate) was synthesized since it should act as a powerful initiator of microtubule assembly. The analog's interactions with tubulin should provide clues to permit exploitation of the exchangeable nucleotide site as a target for microtubule disruption.

Work continued on developing methods to separate the tubulin subunits preparatively and on reconstitution of active tubulin following its denaturation. We continue our efforts to purify a microtubule-associated protein which causes the formation of massive bundles of microtubules.

Publications:

1. Bai R, Duanmu C, Hamel E. Mechanism of action of the antimitotic drug, 2,4-dichlorobenzyl thiocyanate: alkylation of sulfhydryl group(s) of β-tubulin. Biochim Biophys Acta 1989;994:12-20.

2. Banwell MG, Herbert KA, Buckleton JR, Clark GR, Rickard CEF, Lin CM, Hamel E. Synthesis, x-ray crystal structure, and antimitotic properties of 6-chloro-2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)cyclohepta-2,4,6-trien-1-one, a bicyclic analogue of colchicine. J Org Chem 1988;53:4945-52.

3. Batra JK, Kang GJ, Jurd L, Hamel E. Methylenedioxy-benzopyran analogs of podophyllotoxin, a new synthetic class of antimitotic agents that inhibit tubulin polymerization. Biochem Pharmacol 1988;37:2595-602.

4. Duanmu C, Shahrik LK, Ho HH, Hamel E. Tubulin-dependent hydrolysis of guanosine triphosphate as a screening test to identify new antitubulin compounds with potential as antimitotic agents: application to carbamates of aromatic amines. Cancer Res 1989;49:1344-8.

5. Finley RW, Cooney DA, Dvorak JA. Nucleoside uptake in Trypanosoma cruzi: analysis of a mutant resistant to tubercidin. Mol Biochem Parasitol 1988;31:133-40.

6. Hamel E, Ho HH, Kang GJ, Lin CM. Cornigerine, a potent antimitotic Colchicum alkaloid of unusual structure: interactions with tubulin. Biochem Pharmacol 1988;37:2445-9.

7. Itoh Y, Brossi A, Hamel E, Flippen-Anderson JL, George C. Colchicine models: synthesis and antitubulin activity of C(2′)-monosubstituted and C(2′,5)-disubstituted 2,3,4,4′-tetramethoxybiphenyls. Synthesis of 2,3,4,4′-tetramethoxy-biphenyl-2-,5′-dicarboxylic acid. Helv Chim Acta 1989;72:196-204.

8. Itoh Y, Brossi A, Hamel E, Lin CM. Colchicine models: synthesis and binding to tubulin of tetramethoxybiphenyls. Helv Chim Acta 1988; 71:1199-209.

9. Johnson MA, Ahluwalia G, Connelly MC, Cooney DA, Broder S, Johns DG, Fridland A. Metabolic pathways for the activation of the antiretroviral agent 2′,3′-dideoxyadenosine in human lymphoid cells, J Biol Chem 1988;263:1534-7.

10. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim HY, Marquez VE, Johns DG. Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase. J Biol Chem 1989;264:713-8.

11. Lin CM, Singh SB, Chu PS, Dempcy RO, Schmidt JM, Pettit GR, Hamel E. Interactions of tubulin with potent natural and synthetic analogs of the antimitotic agent combretastatin, a structure-activity study. Mol Pharmacol 1988;34:200-8.

12. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Gartner S, Popovic M, Hao Z, Gerrard TL, Wilson YA, Johns DG, Broder S. Inhibition of human immunodeficiency virus (HIV-1/HTLV-III_{Ba-L}) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2′,3′-dideoxynucleosides. J Exp Med 1988; 168:1111-25.

13. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb, DS, Hao Z, Mitsuya, H, Johns DG, Broder S. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3′-azido-2′,3′-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. J Exp Med 1989;169:933-51.

14. Pettit GR, Singh SB, Schmidt JM, Niven ML, Hamel E, Lin CM. Isolation, structure, synthesis and antimitotic properties of combretastatins B-3 and B-4 from Combretrum caffrum. J Nat Prod 1988;51:517-27.

15. Pettit GR, Singh SB, Hamel E, Lin CM, Alberts DS, Garcia-Kendall D. Isolation and structure of the strong cell growth and tubulin inhibitor combretastatin A-4. Experientia 1989;45:209-11.

16. Spigelman Z, Duff R, Beardsley GP, Broder S, Cooney D, Landau NR, Mitsuya H, Ullman B, McCaffrey R. 2′,3′-Dideoxyadenosine is selectively toxic for TdT-positive cells. Blood 1988;71:1601-8.

17. Bai R, Lin CM, Nguyen NY, Liu T-Y, Hamel E. Identification of the cysteine residue of β-tubulin alkylated by the antimitotic agent 2,4-dichlorobenzyl thiocyanate, facilitated by separation of the protein subunits of tubulin by hydrophobic column chromatography. Biochemistry, in press.

18. Bender RA, Hande KR, Hamel E. The plant alkaloids. In: Chabner BA, ed. Cancer chemotherapy: principles and practice, Philadelphia: JB Lippincott Co., in press.

19. Cooney DA, Ahluwalia G, Feldman D, Weinhold P, Baker DC, Hao Z, Lomax N, Hebbler AK, Perno C, Chisena T, Hartman N, Broder S, Johns DG. Characterization of 2′,3′-dideoxycytidine diphosphocholine: a prominent phosphodiester metabolite of 2′,3′-dideoxycytidine. Drug Metab Dispos, in press.

20. Hamel E. Interactions of tublin with small ligands. In: Avila A, ed. Microtubule proteins, Boca Raton: CRC Press, in press.

21. Lin CM, Ho HH, Pettit GR, Hamel E. The antimitotic natural products combretastatin A-4 and combretastatin A-2: studies on the mechanism of their inhibition of the binding of colchicine to tubulin. Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07102-14 LBP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Tubulin as a Site for Pharmacologic Attack

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Hamel	Senior Investigator	LBP, NCI
Others:	R.-L. Bai	Visiting Fellow	LBP, NCI
	Z. Getahun	Visiting Fellow	LBP, NCI
	G. J. Kang	Staff Fellow	LBP, NCI
	C. M. Lin	Biologist	LBP, NCI

COOPERATING UNITS *(if any)* I) G.R. Pettit, Arizona State University; 2) L. Jurd, Dept. of Agriculture; 3) L.J. Powers, Ricerca Corp., Painesville, OH; 4) A. Brossi, NIDDK; 5) M.G. Banwell, Univ. of Melbourne, Australia; 6) Dr. N.Y. Nyugen, FDA; 7) Dr. J.B. Jiang, Dupont Corp.

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unraduced type. Do not exceed the space provided.)*

The goal of this project is the development of new antineoplastic agents directed against tubulin, a protein critical for cell division. Detailed mechanistic and structure-activity studies were performed with six classes of compounds. Combretastatins A-2 and A-4 were found to bind tightly and rapidly, but reversibly, at the colchicine site of tubulin. The pentapeptide dolastatin 10 was found to be a noncompetitive inhibitor of vinca alkaloid binding to tubulin and to prevent nucleotide binding to tubulin. Structure-activity studies were performed with analogs of dolastatin 10, colchicine, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone, and a new synthetic class of antimitotic agents, derivatives of 2-styrylquinazolin-4-one. Colchicine analogs with unusual structural features and/or biological properties were evaluated to quantitate their interactions with tubulin and their mechanism of binding to the protein. Derivatives of 5,6-diphenylpyridazin-3-one, which bind to a distinct site on tubulin (i.e., no competition with other drugs), continued to be evaluated in a search for maximally active agents, and their binding at a unique site on tubulin was examined with radiolabeled preparations.

PHS 6040 (Rev. 1/84) GPO 914-916

Microtubules, cellular organelles critical for cell division, are sensitive to a number of antineoplastic drugs. These drugs all cause cells to accumulate in metaphase, disrupting mitosis, for microtubules form the mitotic spindle. The major constituent of microtubules is an acidic protein known as tubulin, and it is the cellular target of virtually all antimitotic drugs. New antimitotic agents continue to be an active area of interest in the laboratory. We are currently studying the following classes of drugs:

1) Analogs of combretastatin: Combretastatin (NSC 348103) is a natural product isolated by G. R. Pettit of Arizona State University from the South African tree Combretum caffrum; and we have demonstrated that combretastatin is a potent inhibitor of tubulin polymerization and binds at the colchicine site of tubulin. Several still more potent analogs of combretastatin were identified in collaboration with Dr. Pettit's group, and two of these (combretastatin A-2 and combretastatin A-4) were chosen for more detailed studies. We wished to determine the molecular mechanism by which they inhibited binding of radiolabeled colchicine to tubulin by over 90% when present in an equimolar concentration with colchicine. We found that the Combretum caffrum compounds bound rapidly to tubulin even at 0 °C, in contrast to the slow, temperature-dependent binding of colchicine. Although binding of the drugs was tight, the reactions were reversible. Our findings with these drugs, as well as with others, led us to propose a new model for the colchicine site on tubulin.

2) Dr. Pettit has isolated a series of novel cytotoxic peptides from the marine animal Dolabella auricularia. The most active is a pentapeptide, dolastatin 10, which contains four unusual amino acids and potently inhibits microtubule assembly. We have been intensively studying dolastatin 10's interactions with tubulin. It potently inhibits the binding of radilabeled vinca alkaloids to tubulin, but it acts as a noncompetitive inhibitor. Dolastatin 10 also strongly inhibits tubulin-dependent GTP hydrolysis and GTP binding at the exchangeable site of tubulin. Dolastatin 10 does not, however, displace bound nucleotide from tubulin. Dolastatin 10 has nine asymmetric carbon atoms, and Dr. Pettit's group has synthesized approximately fifteen stereoisomers of the peptide, several of which are active, and at least one active tripeptide fragment. These compounds will allow us to define key portions of the dolastatin 10 molecule essential for its cytotoxic properties.

3) Dr. A Brossi of the NIDDK has isolated and synthesized a large number of analogs of the classic microtubule inhibitor colchicine. We have undertaken a collaboration with Dr. Brossi to quantitate more precisely interactions of these analogs with tubulin to provide structure-activity insights into a number of the unique characteristics of the colchicine-tubulin interaction (e.g., temperature-dependent, relatively slow, and irreversible binding of the drug to the protein). An additional goal is the identification of analogs with enhanced potential as antineoplastic agents.

4) Dr. M. G. Banwell of the University of Melbourne synthesized two chlorinated derivatives of the model colchicine analog 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTPT). We found one chlorinated

derivative had enhanced activity, and the other greatly reduced activity. Subsequently Dr. Banwell prepared a series of analogs with modifications in the trimethoxybenzene ring. While none was as active as MTPT, the analogs allowed us to define the relative importance of the three methoxy groups on the phenyl ring.

5) Dr. L. J. Powers of Ricerca Corporation has prepared numerous derivatives of 5,6-diphenylpyridazine-3-one as potential antihypertensive agents. Some of these compounds were found to be potent herbicides as a consequence of inhibition of mitosis in plant tissues. Several members of this class were then submitted to the NCI for screening, and some of these were found to have antineoplastic activity. We have found that a number of these drugs inhibit mitosis in mammalian cells in culture and the polymerization of tubulin in vitro. They potently stimulate tubulin-dependent GTP hydrolysis; but they probably bind at a previously undescribed site on tubulin, for they do not inhibit the binding of colchicine, vinblastine, maytansine or GTP to the protein. Active compounds possess a nitrile group at position 4; and in vitro interactions with tubulin are significantly enhanced by chloride substituents on the phenyl rings, both of which are required for antitubulin activity. There is little overlap between compounds most active against mammalian tubulin and those which are most active in inhibiting mitosis in plant cells. In collaboration with Dr. Powers we are continuing to study structure-activity correlations in this class of drugs to develop maximally active agents. Two active compounds have been prepared in a radiolabeled form, one with the label in the phenyl rings, the other with the radiolabel in the pyridazinone ring. We have had difficulty in demonstrating binding of either radiolabeled drug to tubulin, indicating that the drug-protein complex is unstable, but recently we have succeeded in precipitating a drug-tubulin complex. We are now attempting to develop a reliable assay to study effects of other agents on complex formation.

6) Dr. J. B. Jiang of the Dupont Corporation has synthesized a series of derivatives of 2-styrylquinazolin-4-one with antineoplastic activity. The most active members of the series strongly inhibit tubulin polymerization, and we are performng a structure-activity analysis of these compounds. Detailed studies of their cytotoxicity and mechanism of binding to tubulin will be performed.

Publications:

1. Hamel E, Ho HH, Kang GJ, Lin CM. Cornigerine, a potent antimitotic Colchicum alkaloid of unusual structure: interactions with tubulin. Biochem Pharmacol 1988;37:2445-9.

2. Pettit GR, Singh SB, Schmidt JM, Niven ML, Hamel E, Lin CM. Isolation, structure, synthesis and antimitotic properties of combretastatins B-3 and B-4 from Combretrum caffrum. J Nat Prod 1988;51:517-27.

3. Batra JK, Kang GJ, Jurd L, Hamel E. Methylenedioxy-benzopyran analogs of podophyllotoxin, a new synthetic class of antimitotic agents that inhibit tubulin polymerization. Biochem Pharmacol 1988;37:2595-602.

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10. Itoh Y, Brossi A, Hamel E, Flippen-Anderson JL, George C. Colchicine models: synthesis and antitubulin activity of C(2′)-monosubstituted and C(2′,5)-disubstituted 2,3,4,4′-tetramethoxybiphenyls. Synthesis of 2,3,4,4′-tetramethoxy-biphenyl-2-,5′-dicarboxylic acid. Helv Chim Acta 1989;72:196-204.

11. Bai R, Lin CM, Nguyen NY, Liu T-Y, Hamel E. Identification of the cysteine residue of β-tubulin alkylated by the antimitotic agent 2,4-dichlorobenzyl thiocyanate, facilitated by separation of the protein subunits of tubulin by hydrophobic column chromatography. Biochemistry, in press.

12. Lin CM, Ho HH, Pettit GR, Hamel E. The antimitotic natural products combretastatin A-4 and combretastatin A-2: studies on the mechanism of their inhibition of the binding of colchicine to tubulin. Biochemistry, in press.

13. Hamel E. Interactions of tublin with small ligands. In: Avila A, ed. Microtubule proteins, Boca Raton: CRC Press, in press.

14. Bender RA, Hande KR, Hamel E. The plant alkaloids. In: Chabner BA, ed. Cancer chemotherapy: principles and practice, Philadelphia: JB Lippincott Co., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07179-04 LBP

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October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein-protein and Protein-nucleotide Interactions in Microtubule-Assembly

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	E. Hamel	Senior Investigator	LBP, NCI
OTHERS:	Z. Getahun	Visiting Fellow	LBP, NCI
	G. J. Kang	Staff Fellow	LBP, NCI
	C. M. Kin	Biologist	LBP, NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.7	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The rational development of new antineoplastic agents directed against tubulin, a protein critical for cell division, requires greater understanding of the interaction between the polypeptide subunits of tubulin, its two tightly bound guanine nucleotides, and microtubule-associated proteins. The effects of nucleotides on the stability of microtubules continued to be examined, as were conditions to optimize the separation of α-tubulin and β-tubulin on a preparative scale. The purification of a microtubule-associated protein which causes the formation of microtubule bundles continued to progress. Projects to define the cystine bridges in tubulin and microtubule-associated proteins and to introduce potentially antimitotic nucleotide analogs into cells continued. Polymerization reactions supported by nonhydrolyzable-GTP analogs and ATP were reevaluated. Roles of divalent cations in nucleotide binding to tubulin and in tubulin polymerization were examined.

PHS 6040 (Rev 1/84)

GPO 914-918

Microtubules, cellular organelles critical for cell division, are sensitive to a number of antineoplastic drugs. Their major constituent is an acidic protein known as tubulin, which consists of two different polypeptide chains and two molar equivalents of guanine nucleotide. Half this nucleotide (the exchangeable nucleotide) is in the form of either GTP or GDP. If GTP, it is hydrolyzed to GDP during microtubule assembly from tubulin and microtubule-associated proteins (MAPs -- minor, but essential, components of the microtubule). The remainder of the nucleotide exists only as GTP (the nonexchangeable nucleotide). This GTP is not altered by destroying the protein. Its function is unknown.

It has long been believed that nonhydrolyzable GTP analogs can support tubulin polymerization, although not as efficiently as GTP. This view was recently challenged, and we decided that it required careful reinvestigation. We concluded that the original findings were correct, but that polymerization reactions with nonhydrolyzable analogs were exquisitely sensitive to reaction conditions.

We are quantitatively investigating effects of Mg^{2+} on the relative affinities of GDP and GTP for tubulin, extending our earlier finding that Mg^{2+} was required for the binding of GTP, but not GDP, to tubulin. We have also confirmed a report of another laboratory, that Be^{2+}, as a complex with F^-, stabilizes glycerol-induced tubulin polymers. With MAPs, however, Be^{2+} alone is effective, appearing to replace Mg^{2+} in microtubule formation, and F^- inhibits this effect. This is a useful observation, because a radioactive isotope of Be^{2+} is commercially available. This should permit us to follow the fate of Be^{2+} as a Mg^{2+} analogue in nucleotide binding and in microtubule assembly.

We have continued to study the stability of microtubules as a function of their nucleotide content and environment. In particular, we are trying to determine whether stability is affected by the proportion of microtubule nucleotide which has resulted from hydrolysis of GTP to GDP as opposed to the proportion of GDP incorporated directly into the polymer. A related issue is the observation that microtubule integrity requries some GTP in the reaction mixture, for microtubules rapidly disintegrate if GTP is totally degraded by transfer of the terminal phosphate to fructose-6-phosphate by phosphofructokinase. Although we have established that nonexchangeable GTP is not destroyed in this reaction, we have yet to localize unambiguously the essential triphosphate.

We plan to exploit the known ability of guanosine 5′-[α-β-methylene]-triphosphate to disrupt microtubule organization when injected into cells and the similar in vitro behavior of this analog and 2′,3′-dideoxyguanosine 5′-triphosphate with tubulin. We are attempting to design antimitotic GTP analogs able to penetrate cells. We plan the synthesis of analogs with reduced phosphate charge and hydrophobic modifications at position 8 of the guanine ring. These will contain the dideoxyribose and/or α-β methylene modifications.

For many years we have been attempting to reproducibly and preparatively separate the two subunits of tubulin. Although we had achieved significant separation by hydrophobic chromatography, reproducibility has been a problem. Continued efforts in the past year have not been successful. One goal of this

project is reconstitution of activity from the separated subunits plus small ligands (i.e., GTP and/or GDP and MG^{2+}). Even in the absence of a totally successful separation of subunits, we have begun to search for conditions to reactivate denatured tubulin, using as a starting point previously determined reaction conditions in which tubulin is optimally stable.

We are utilizing the ability to separate the a-and β-tubulin subunits to locate the protein's cystine disulfide bridges. Our strategy is to alkylate with nonradiolabeled sulfhydryl alkylating reagent, reduce, and alkylate with radiolabeled reagent. The protein will then be degraded with cyanogen bromide and the radiolabeled peptides purified and sequenced. Similar studies are planned with MAPs.

We are continuing to devote a great deal of attention to MAPs. We are particularly interested in a MAP which causes the formation of microtubule bundles (distinct microtubules which aggregate laterally). The active component (termed MAP-TB) appears to be present in MAP preparations in extremely small amounts. Although it is highly stable, it has proven more difficult to purify than anticipated. Despite DEAE-cellulose chromatography, ammonium sulfate fractionation, heat-treatment, hydroxyapatite chromatography, and HPLC chromatography (ion-exchange and gel filtration), the purest preparations remain disappointingly heterogeneous on polyacrylamide gel electrophoresis. Tubulin affinity chromatography may be useful in its purification.

Publications:

1. Duanmu C, Shahrik LK, Ho HH, Hamel E. Tubulin-dependent hydrolysis of guanosine triphosphate as a screening test to identify new antitubulin compounds with potential as antimitotic agents: application to carbamates of aromatic amines. Cancer Res 1988;49:1344-8.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07181-04 LBP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical and Pharmacological Studies with Chemotherapeutic Nucleosides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	D. Cooney	Senior Investigator	LBP, NCI
Others:	Z. Hao	Visiting Scientist	LBP, NCI
	Y. Wilson	Chemist	LBP, NCI
	N. Hartman	Staff Fellow	LBP, NCI
	R. Masood	Visiting Fellow	LBP, NCI

COOPERATING UNITS *(if any)*

C. Perno, R. Yarchoan, D. Poplack and S. Broder, COP, DCT, NCI, Christina Chisena, LBC, DTP, DCT, NCI, A. Fridland, St. Jude Childrens' Research Hospital, Memphis, TN and David Baker, University of Alabama

LAB/BRANCH

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SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues X☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Zebularine is a pyrimidone with substantial activity against L1210 leukemia. The compound is an effective inhibitor of cytidine deaminase but inasmuch as L1210 lacks this enzyme, its inhibition is not likely to be involved in the antineoplastic effects of Zebularine. During the past year we have examined the metabolism of Zebularine in L1210 cells and in the human lymphoblastic line, Molt-4: Both lines convert the drug to acidic products with the properties of 5′-mono, di and triphosphates. A pilot sample of Zebularine-5′-phosphate was found to be a potent inhibitor of deoxycytidylate deaminase with a K_i of 200 nM. This metabolite might be responsible for the increases (10-20 fold) of dCTP which ensue upon exposure of L1210 and Molt-4 cells to Zebularine. The cellular pharmacology of several novel dideoxynucleosides with antiretroviral activity has been examined in a variety of human and murine lines. It has been found that 2′,3′-dideoxy-2′-fluoroarabinosyl adenine (fddA), a sugar-halogenated analog of 2′,3′-dideoxyadenosine (ddA) with markedly enhanced stability to acid, also exhibits markedly enhanced metabolic stability; thus fddA is deaminated at a significantly reduced rate vis-á-vis ddA; more importantly, the resultant deamination product 2′3′-dideoxy-2′-fluoroarabinosyl inosine (fddI) demonstrated total resistance to phosphorylytic attack by purine nucleoside phosphorylase. In addition, the 5′-monophosphate of fddA, fddAMP, is, of the series of purines examined, the most resistant to deamination by adenylate deaminase. In Molt-4, CEM and ATH-8 cells, the combined chemical and metabolic stability of fddA translates into a marked enhancement in the degree of accumulation of fddADP (22 fold) and of fddATP (5 fold) after short term exposure to 10 μM drug. These results warrant the expectation that fddA could provide significant pharmacological advantages over the prototype drug, ddA, and might be useful for oral administration to patients with AIDS.

1. Studies with Zebularine

Zebularine is a pyrimidone ribonucleoside with excellent activity against both the intraperitoneal and subcutaneous L1210 tumor models. The drug is an effective inhibitor of cytidine deaminase, with a K_i versus the mouse kidney enzyme of ca. 1×10^{-7} M. However, inasmuch as L1210 cells are practically devoid of cytidine deaminase, this enzyme is believed not to be the molecular target for Zebularine. During the past year, we have compared the pharmacological properties of Zebularine with those of two second-generation congeners: 2′-fluorozebularine and 5-fluorozebularine. All three agents are submicromolar inhibitors of cytidine deaminse; in addition, as their 5′-monophosphates, all are effective inhibitors of murine and human deoxycytidylate deaminase. In vitro, these inhibitions translate into expansions of the cytidine, dCMP, and finally dCTP pools. Inasmuch as dCTP is a powerful inhibitor of deoxycytidine kinase, its accumulation - which reaches 20-fold in Zebularine-treated lymphoblasts - represses the efficient operation of this kinase and effectively blocks phosphorylation of deoxycytidine or dideoxycytidine. Using [^{14}C]zebularine, we have established that the drug is converted to a family of acidic species in L1210 and Molt-4 cells; these are presumed to be the 5′-mono, di and triphosphates. Although Molt-4 cells have been found to be resistant, in vitro, to the cytotoxic actions of Zebularine (IC_{50} > 100 μM), the aforementioned metabolites accumulated to equivalent concentrations in these, and in L1210 cells.

Surprisingly, the macromolecular incorporation of thymidine, uridine and L-valine were not strongly repressed by Zebularine in either Molt-4 or L1210 cells, in vitro. This result, if confirmed in vivo, leaves open the mechanism by which this pyrimidone exerts its antiproliferative activity.

2. Studies with 2′3′-Dideoxy-2′-fluoroarabinosyl adenine

2′,3′-Dideoxyadenosine (ddA) and its deamination product 2′,3′-dideoxyinosine (ddI) are useful antiretroviral drugs in man, but their chemical and metabolic instability (as detailed in last year's annual report) militate against their optimal use in the clinic. As a first step in circumventing this problem, chemists within the Laboratory of Medicinal Chemistry, DTP, have synthesized 2′,3′-dideoxy-2′-fluoroarabinosyl adenine (fddA) and demonstrated that its glycosidic linkage is, in marked contrast to that of ddA, wholly resistant to attack by acid; in addition, studies from the Toxicology Branch have established that fddA exhibits excellent bioavailability in the dog, a feature which doubtless reflects its stability to gastric acid. On this background we have begun an examination of the cellular pharmacology of fddA using tritiated drug as marker and human lymphoblasts (Molt-4, ATH8 and CEM) as our test system. Like ddA, fddA appears to enter cells by passive diffusion at a rate of 9.7 pmoles/10^6 cells/min; fddI, the deamination product of fddA, enters cells at <10% of this rate, a result which is probably a consequence of the lower lipid solubility of the hypoxanthine moiety.

As a first stage in our examination of the metabolic stability of fddA, the fate of the drug (5 μM) was examined in complete RPMI 1640 (containing 10% calf serum, 56° for 1 hr) containing Molt-4 cells in logarithmic growth; parallel flasks received ddA for purposes of comparison. Under these conditions, fddA

was converted to fddI (presumably via adenosine deaminase) at a rate of 1 μmole/hr; in other words the half-life ($t_{1/2}$) approximated 2.5 hrs. No hypoxanthine was detected. In marked contrast, ddA was deaminated to ddI with a $t_{1/2}$ of 10 minutes and abundant hypoxanthine accumulated. Inside the cells, these rates were somewhat faster than in medium, but the results were quantitatively similar, with fddA exhibiting markedly greater stability than ddA. Obviously our failure to detect hypoxanthine in either cells or media incubated with fddA, suggested that, unlike ddI, fddI was not susceptible to phosphorolysis. A direct examination of this point, using an excess of purified splenic purine nucleoside phosphorylase (PNP) confirmed our supposition: even when xanthine oxidase was used to drive the reaction to completion, no spectrophotometric evidence for the phosphorolysis of fddI was obtained. HPLC confirmed this point.

Since fddA exhibited good metabolic stability, we next examined the possibilty that anabolites of the drug would accumulate intracellularly at an enhanced rate vis-á-vis those of ddA. In fact, there was a 22-fold enhancement of the 5′-diphosphate of fddA and a 5-fold enhancement of its 5′-triphopshate in Molt-4 cells incubated for 24 hrs with 5 μM drugs. Inasmuch as fddA is as active as ddA in the ATH8 assay system versus the human immunodeficiency virus (HIV), but exhibits greatly enhanced chemical and metabolic stability, its use in patients with AIDS should be given serious consideration - especially when peroral administration is envisioned.

Several additional enzymologic properties of fddA have also been examined during the past year. Using chemically synthesized fddAMP, we have demonstrated that the fluorinated dideoxynucleoside is an exceedingly poor substrate for adenylate deaminase, a result which reinforces our contention that the drug offers a substantially improved metabolic stability profile over ddA (and ddAMP). Such stability of fddAMP probably contributes to the markedly enhanced accumulation of fddADP and fddATP described earlier, and indicates that a wasteful buildup of fddIMP (the product of deamination at the nucleotide level) should not occur in cells incubated with fddA. This is fortuitous inasmuch as fddIMP is a poor substrate for adenylosuccinate synthetase compared to IMP and dIMP (but roughly equivalent in V_{max} to ddIMP). The in vitro finding of demonstrable, but slow substrate activity of fddIMP (and ddIMP) with IMP dehydrogenase raises the possibility that these metabolites could be converted to guanine dideoxynucleotides intracellularly; indeed HPLC analysis of Molt-4 cells incubated with tritiated fddA reveals the presence of a peak eluting well after fddATP, at a level of approximately 5% of fddATP: this peak is presumably fddGTP.

Lastly, the ability of fddATP to inhibit the reverse transcriptase from HIV was examined in vitro. Using poly (dA-dT) as template-primer, an IC_{50} of 0.15 μM was established. This value is comparable to that of many dideoxynucleosides versus this polymerase.

3. Studies with Congeners of Azidothymidine

Although AZT is a useful drug against AIDS, it does produce significant toxicities in man, most notable among them being damage to the bone marrow. We have, therefore, sought second-generation congeners of AZT and attempted to

intercompare their myelotoxicity and metabolism *in vitro* as a basis for possible clinical development. The two nucleosides selected for this purpose were dideoxythymidine (d2T) and dideoxythymidinene (d4T), both of which differ from AZT by virtue of the absence of a bulky substituent on the 3′-carbon atom.

Table 1. Percent Suppression of Human Hematopoietic Progenitor Cells by AZT, d4T and d2T

Agent	Drug Concentration (M) 10^{-7}	10^{-6}	10^{-5}	10^{-4}
	Mean ± SEM			
	CFU-GM[1]			
AZT	15.5 ± 11.5	22 ± 2	46 ± 12	96.5 ± 0.5
d4T	18.5 ± 7.5	12 ± 4	8.5 ± 8.5	35.5 ± 0.5
d2T	20.5 ± 14.5	16 ± 16	6 ± 6	12.5 ± 12.5
	BFU-E[2]			
AZT	51.5 ± 1.5	71 ± 1	95.5 ± 1.5	99.5 ± 0.5
d4T	7.5 ± 7.5	7.5 ± 7.5	9 ± 0	38.5 ± 3.5
d2T	7 ± 0	9.5 ± 8.5	8 ± 4	9 ± 9

[1]GM colonies were stimulated by 15% placental conditioned medium. Colonies were counted at 10 days. The control CFU-GM was 93 ± 13 per 10^5 mononuclear cells.

[2]BFU-E colonies were stimulated by 1% T cell conditioned medium (MOCM) and 0.5 Unit/ml erthropoietjn. Colonies were counted at 14 days. The control BFU-E was 217 ± 65 per 10^5 mononuclear cells.

Table 1 summarizes the impact of AZT, d2T and d4T on granulocytic colony forming units (CFU-GM) and erythroid burst forming units (BFU-E) from normal human marrow. It can be seen from these data, that AZT is the most toxic of the agents to both of these progenitors, and that the erythroid progenitor is most sensitive to its toxicity, the IC_{50} being 1 x 10^{-7} M; d4T, while less toxic than AZT, nevertheless exerted significant toxicity to the erythroid progenitor population, with an IC_{50} of approximately 1 x 10^{-5} M. D2T, by contrast, was virtually without toxicity to either stem cell population.

We next intercompared the metabolism of these three congeners of thymidine in exponentially growing cultures of Molt-4 cells. In confirmation of the work of others, we documented that AZT showed a strong tendency to accumulate as its 5′-monophosphate. (Not shown is the finding that AZT-5′-monophosphate was

extruded from these cells as such.) When the levels of the presumptive active species - the 5′-triphosphates were measured - AZT, d2T and d4T were found to have been anabolized to an approximately equal extent up until 8 hr of incubation - but thereafter d2TTP underwent a nearly total degradation despite the continuous presence of drug in the medium, a result which may reflect chemical instability under these experimental conditions. Metabolic instability was monitored by measuring the appearance of thymine in the cells over time. Significant thymine was generated from d4T, little from d2T and none from AZT. Presumably any thymine found arises as a consequence of phosphorolysis.

Lastly, several enzymologic properties of the 5′-mono and triphosphates of these three congeners were examined in vitro. It was found that the monophosphates of all 3 nucleosides inhibited thymidylate kinase, but that AZT-5′-MP was most potent in this regard. Versus HIV reverse transcriptase, d4T-5′-TP was about 3 times more potent than d2T-5′-TP but only twice as potent as AZT-5′-TP. Since levels of d4T-5′-TP well above its K_i for reverse transcriptase are formed in Molt-4 cells incubated with 4 μM drug, and since it fails to engender a build-up as the potentially toxic 5′-monophosphate, consideration should be given towards the clinical development of this unsaturated analog of thymidine.

Publications:

1. Johnson MA, Ahluwalia G, Connelly MC, Cooney DA, Broder S, Johns DG, Fridland A. Metabolic pathways for the activation of the antiretroviral agent 2′,3′-dideoxyadenosine in human lymphoid cells, J Biol Chem 1988;263:1534-7.

2. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Gartner S, Popovic M, Hao Z, Gerrard TL, Wilson YA, Johns DG, Broder S. Inhibition of human immunodeficiency virus (HIV-1/HTLV-III_{Ba-L}) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2′,3′-dideoxynucleosides. J Exp Med 1988; 168:1111-25.

3. Spigelman Z, Duff R, Beardsley GP, Broder S, Cooney D, Landau NR, Mitsuya H, Ullman B, McCaffrey R. 2′,3′-Dideoxyadenosine is selectively toxic for TdT-positive cells. Blood 1988;71:1601-8.

4. Finley RW, Cooney DA, Dvorak JA. Nucleoside uptake in Trypanosoma cruzi: analysis of a mutant resistant to tubercidin. Mol Biochem Parasitol 1988;31:133-40.

5. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim HY, Marquez VE, Johns DG. Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase. J Biol Chem 1989;264:713-8.

6. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb, DS, Hao Z, Mitsuya, H, Johns DG, Broder S. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3′-azido-2′,3′-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. J Exp Med 1989;169:933-51.

ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The objectives of the Laboratory of Medicinal Chemistry (LMC) are the discovery and development of new anticancer, anti-AIDS and antiviral drugs. Organic, analytical and polypeptide chemistry as well as computer-assisted molecular modeling research are the areas emphasized to accomplish LMC goals.

Arabinosyl-5-azacytosine (ara-AC), a compound synthesized a number of years ago in this laboratory, completed Phase I clinical trials at several institutions, including both an adult and pediatric study at the NIH Clinical Center. The LMC applied the analytical methods it had developed for measuring ara-AC in body fluids to conduct pharmacokinetic studies in conjunction with both COP clinical trials. Although ara-AC is hydrolytically unstable and rapidly cleared from the plasma, steady-state levels of this drug were found to be proportional to dose and within the range (0.3-1.0 μM) reported for <u>in vitro</u> activity. This later result correlates with the encouraging signs of antitumor activity observed in the adult study.

LMC interest in the antitumor and antiviral activity of cyclopentenyl nucleosides continues to be high based on the continuing program of preclinical development of our analogue, cyclopentenyl cytosine (CPE-C) as a clinical candidate. Pharmacokinetic studies have been carried out in rats and dogs and presently continue in rhesus monkeys using automated HPLC methodology developed in this laboratory. The long terminal phase of elimination ($t_{1/2}$ = 19-37 hr) indicates a deep compartment in which high concentrations of CPE-C or its phosphorylated metabolites might reside.

Analogues of CPE-C which might have different biochemical properties have been prepared. 3-Deaza-CPE-C, 3-deaza-CPE-U (which is also a 3-deazauridine analogue) and 5-aza-CPE-C have been synthesized and are being evaluated for antitumor and antiviral activity. 3-Deazaneplanocin, synthesized earlier by us, has been found to have the highest selectivity index ever observed against rotavirus (10,000). A successful total synthesis of neplanocin F, a naturally occurring isomer of neplanocin A, was accomplished.

Analytical methodology has been developed in this laboratory to determine the bioavailability of oral HMBA in Phase I clinical studies and to compare disposition and metabolism between oral and parental dosage forms. A gas chromatographic method allows simultaneous measurement of HMBA and its major acid and basic metabolites. We have used this methodology to show that the oral drug in solution is completely bioavailable and that tablet HMBA is bioequivalent to solution HMBA in terms of plasma levels, metabolic pattern and urinary excretion.

Among the initial compounds synthesized as designed inhibitors of protein kinase C, several have been shown to be specific inhibitors of the binding of [^{3}H]phorbol-12,13-dibutyrate in the mouse cytosolic phorbol aporeceptor assay (ID_{50}=8 μg/ml).

The LMC's unique phosphoramidite synthon (containing a stabilized form of 5-azacytidine) has been used to prepare sequence specific 26-mers using a DNA synthesizer. These materials are being evaluated for their ability to inhibit DNA methylase and cause altered gene expression.

2'-Deoxy-2'-fluoro-2-oxopyrimidine riboside (2'-F-zebularine) was synthesized as an intermediate in the synthetic pathway to the corresponding 3'-deoxy analogue, intended for anti-HIV testing. It was discovered in collaboration with the Laboratory of Biochemical Pharmacology that 2'-F-Zeb is a potent and selective inhibitor of dCMP deaminase. A compound with this mechanism of action could be useful when administeed with anticancer as well as anti-AIDS drugs which are deaminated at the nucleotide level to inactive metabolites.

Oxetanocin is a naturally occurring anti-AIDS nucleoside containing an unusual four-membered oxetane "sugar" ring. In an attempt to provide information on whether the 4-member ring was crucial for activity, two ring-expanded isomers were synthesized. One was active, one was not. Additional work is on-going to further elucidate the structure-activity relationships in this series.

Six 2'-fluoro, acid-stable dideoxyadenosine nucleosides were synthesized in an attempt to increase the partition coefficient and enhance the CNS penetration of the anti-AIDS parent compound, 2'-F-dd-ara-A. Methyl substitution at either the 2- or 8-positions on the purine ring abolished activity. However, methyl or benzoyl substitution on the 6-amino group resulted in more lipophilic analogues which retained ca. 75% of the protective effect of ddA. Structure-activity studies are in progress.

Molecular modeling using the hardware and software available at DCRT has provided structural insights into possible critical structural parameters found in active anticancer and anti-AIDS compounds. This technique has been extended in a prospective manner to current LMC synthetic research related to inhibitors of protein kinase C, dideoxypurine nucleosides and oxetanocin analogues.

A polypeptide laboratory has been established and an anti-AIDS project has been designed. The objective is the discovery of new anti-HIV drugs by the inhibition of the retroviral protease. The approach being used is based on the anti-sense polypeptide concept.

Publications:

1. Benjamin T, Niu C-H, Parmellee DC, Huggett Ac, Yu B, Roller PP and Thorgeirsson SS. Direct N-terminal sequence analysis of rat liver plasma membrane glycoproteins separated by two dimensional polyacrylamide gel electrophoresis, Electrophoresis, in press.

2. Collins JM, Klecker RW Jr., Kelley JA, Roth JS, McCully CL, Balis FM Poplack DG. Pyrimidine dideoxyribonucleosides: Selectivity of penetration into cerebrospinal fluid, J Pharmacol Exp Ther 1988;245:466-70.

3. De Clercq E, Cools M, Balzarini J, Marquez VE, Borcherding DR, Borchardt RT, Drach JC, Kitaoka S, Konno T. Broad-spectrum antiviral activity of neplanocin A, 3-deazaneplanocin A and 5′-Nor derivatives thereof, Antimicrob Ag Chemother, in press.

4. Driscoll JS, Marquez VE, Plowman J. Cyclopentenyl cytosine (CPE-C). A carbocyclic nucleoside with antitumor and antiviral properties, Nucleosides Nucleotides, in press.

5. Driscoll JS, Khan AH, Chou F-T. US Patent 4,704,384: Aziridinyl quinone antitumor agents, November 3, 1987.

6. Driscoll JS, Marquez VE, Kim C-H, Kelley JA. US Patent 4,788,181: 5-Substituted-2′,3′-dideoxycytidine compounds with anti-HTLV-III activity, November 29. 1988.

7. Fales HM, Blum MS, Southwick EK, Williams DL, Roller PP and Don AW. Structure and synthesis of tetrasubstituted pyrazines in ants in the genus Mesoponera, Tetrahedron 1988;44:5045-5050.

8. Fassina G, Roller PP, Olson AD, Thorgeirsson SS and Omichinski JG. Recognition properties of peptides hydropathically complementary to residues 356-375 of the c-raf protein, J Biol Chem, in press.

9. Goddard AJ, Marquez VE. Synthesis of oligonucleotides containing 5,6-dihydro-5-azacytosine and 5-azacytosine at specific CpG sites, Nucleosides Nucleotides, in press.

10. Goldstein BM, Bell JE, Marquez VE, Robins RK. Dehydrogenase binding by the cofactor analogue TAD. Molecular Structure. Chemical Reactivity and Biological Activity. International Union of Crystallography Crystallographic Symposia No 2. Stezowski JJ, Huang J-L, Shao M-C. eds. Oxford Univ Press New York 1988 pp 163-168.

11. Han K-H, Niu, C-H, Roller PP and Ferretti JA. Conformation of the second disulfide loop in human transforming growth factor α studied by two-dimensional NMR spectroscopy, Biopolymers 1988; 27: 923-937.

12. Heideman RL, Roth JS, Ford H Jr., Kinnard RD, Litterst CL, Kelley JA. Reverse phase HPLC determination and murine pharmacokinetics of arabinosyl-5-azacytosine, J Liquid Chromatogr, in press.

13. Heideman RL, Gillespie A, Ford H, Reaman GH, Balis FM, Tan C, Sato J, Ettinger LW, Packer RJ, Poplack DG. Phase I trial and pharmacokinetic evaluation of fazarabine in children, Cancer Res, in press.

14. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim H-Y, Marquez VE, Johns DG. Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase, J Biol Chem 1989;264:713-18.

15. Kassianides C, Hoofnagle JH, Miller RH, Doo E, Ford H, Broder S, Mitsuya H. Inhibition of duck hepatitis virus replication by 2′,3′-dideoxycytidine. a potent inhibitor of reverse transcriptase, J Gastroenterology, in press.

16. Kern DH, Driscoll JS, Hildebrand-Zanki SU, Shoemaker RH. Structure-activity studies of catechol analogs with selective toxicity for malignant melanoma. Progress in chemotherapy, Berkarda B, Kuemmerle H-P. eds. Ecomed Landsberg, 1988; pp 585-587.

17. Kern DH, Shoemaker RH, Hildebrand-Zanki Su, Driscoll JS. Structure-activity relationships defining the cytotoxicity of catechol analogues against human malignant melanoma, Cancer Res 1988;48:5178-5182.

18. Marquez VE, Lim M-1, Treanor SP, Plowman J, Priest MA, Markovac A, Khan, MS, Kaskar B, Driscoll J. Cyclopentenylcytosine. A carbocyclic nucleoside with antitumor and antiviral properties, J Med Chem 1988; 31:1687-1694.

19. Marquez VE, Lim M-I, Tseng CKH, Markovac A, Priest MA, Khan SM, Kaskar, B. Total Synthesis of Neplanocin A. J Org Chem 1988;53:5709-5714.

20. Marquez VE, Lim M-I, Khan MS, Kaskar B. (4R,5R)-(-)-3-Benzyloxymethyl-4,5-O-isopropylidene-2-cyclopentenone. An optically active α,β-Unsaturated cyclopentenone for the synthesis of neplanocin A and other cyclopentene carbocyclic nucleosides. In Townsend LB and Tipson RS. eds. Nucleic Acid Chemistry. Improved and new synthetic procedures, methods and techniques Part 3, in press.

21. Marquez VE, Lim M-I, Markovac A, Priest MA. (-)-Neplanocin A. In Townsend LB and Tipson RS. eds. Nucleic Acid Chemistry. Improved and new synthetic procedures, methods and techniques. Part 3, in press.

22. Marquez VE. The Design, Synthesis, and Antiviral Activity of Nucleoside and Nucleotide Analogues. In ACS Symposium Series. Antiviral Agents, Martin JC. ed., in press.

23. Marquez VE. Effect of fluorine substitution on the anti-HIV activity of dideoxynucleosides. In NIDA Research Monographs, in press.

24. Moon D-C, Kelley JA. A simple desalting procedure for fast atom bombardment mass spectrometry. Biomed Environ Mass Spectrom 1988;17:229-37.

25. Milne GWA. Use of a CENTRUM workstation in a drug design laboratory. Proceedings of the London Annual Online Meeting. 1988;12:189-201 .

26. Milne GWA. Molecular Modelling in Drug Design. Proceedings of the Montreux 1989 International Chemical Information Conference, in press.

27. Niu, C-H, Han K-H and Roller PP. Comparison of the conformation and GTP hydrolysing ability of N-terminal ras $_{D}$21 protein segments. Biochem Biophys Res Commun. 1989;160:282-288.

28. Tseng CKH, Marquez VE, Fuller RW, Goldstein BM, Haines DR, McPherson H, Parsons JL, Shannon WM, Arnett G, Hollingshead M, Driscoll JS. Synthesis of 3-deazaneplanocin A: A powerful inhibitor of S-adenosyl homocysteine hydrolase with potent and selective in vitro and in vivo antiviral activities. J Med Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 03580-20 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Research in the Development of New Anticancer Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

J. S. Driscoll		Chief	LMC, NCI
Others:	V. E. Marquez	Deputy Chief	LMC, NCI
	J. A. Kelley	Research Chemist	LMC, NCI
	L. Hegedus	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

Pediatric Oncology Branch, Medicine Branch, COP, DCT;
Pharmacology Branch, Laboratory of Biochemical Pharmacology, DTP, DCT.

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ara-AC, an LMC drug, has completed Phase I clinical testing by the COP in Building 10 and by two other institutions. This compound showed signs of antitumor activity against several tumor types during these dose-finding studies.

Cyclopentenyl cytosine (CPE-C) has DN-2B status in the DCT Decision Network process and is currently undergoing toxicological evaluation by DTP. Mouse, rat, dog and monkey studies using an analytical method developed in the LMC show an unusual toxicity profile which may be related both to a long-lived triphosphate metabolite and cytosine induction in treated animals. Toxicity is particularly schedule-dependent. Pharmacokinetic modeling by Dr. Zaharko was initiated in an attempt to produce a method to predict cross-species toxicity.

Publications:

1. Driscoll JS, Khan AH, Chou F-T. US Patent 4,704,384: Aziridinyl quinone antitumor agents, November 3, 1987.

2. Kern DH, Shoemaker RH, Hildebrand-Zanki SU, Driscoll JS. Structure-activity relationships defining the cytotoxicity of catechol analogues against human malignant melanoma, Cancer Res, 1988;48:5178-5182.

3. Kern DH, Driscoll JS, Hildebrand-Zanki SU, Shoemaker RH. Structure-activity studies of catechol analogs with selective toxicity for malignant melanoma. In: Berkarda B, Kuemmerle H-P, eds. Chemotherapy. Landsberg: Ecomed, 1988; 585-7.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06173-04 LMC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Dideoxynucleosides as Potential Anti-AIDS Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor E. Marquez	Research Chemist	LMC, NCI
Others: J. S. Driscoll	Chief	LMC, NCI
J. Barchi	Senior Staff Fellow	LMC, NCI
R. Wysocki	IRTA Fellow	LMC, NCI

COOPERATING UNITS *(if any)*
Toxicology Branch, DTP, DCT, NCI
DTP Anti-HIV Testing Program, FCRF
Office of the Associate Director, Clinical Oncology Program, DCT, NCI.

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.25	0.25

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The syntheses of the acid stable 2′-F-dd-ara-A and 2′-F-dd-ara-I have been improved and extended to other purine and pyrimidine members. 2′-F-dd-ara-I proved to be as effective as its parent and is completely resistant to both acid and enzymatic degradation by purine nucleoside phosphorylase.

Efforts toward the augmentation of the lipophilic character of 2′-F-dd-ara-A resulted in the syntheses of analogues bearing a methyl substituent at positions 2-, 8-, and N-6 of the purine ring. Only the N-6 methyl compound maintained significant anti-HIV activity. This lead will be investigated.

Synthesis of five-member ring analogues of the oxetane-containing natural product oxetanocin, demonstrated that the four-member oxetane ring is not necessary for anti-HIV activity. Only the 3′-hydroxymethyl isomer of the two possible ring-enlarged analogues demonstrated significant anti-HIV activity, The activity was indistinguishable from that of oxetanocin. The synthesis of several analogues in this series is in progress.

PHS 6040 (Rev 1/84) GPO 914-918

Project Description:

General Objective:

The objective of this project is the discovery of 2′,3′-dideoxynucleoside analogues superior to known inhibitors of the AIDS virus.

Specific Objectives:

I. Synthesis of acid-stable purine and pyrimidine analogues.

2. Synthesis of potentially CNS-active purine analogues with increased lipophilicity.

3. Synthesis of ring-enlarged analogues of oxetanocin.

Major findings:

Synthesis of Acid-Stable Dideoxypurine Nucleosides (Dr. Marquez, Dr. Barchi, Dr. Driscoll):
The syntheses of acid-stable and anti-HIV active dideoxynucleosides, 9-(2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl)-adenine and hypoxanthine [2′-fluoro-ara-ddA (1) and 2′-fluoro-ara-ddI (2), respectively] were improved. Extensive biological and pharmacological studies continue. Like ddA, 2′-fluoro-ara-ddA (1) is readily converted to the inosine derivative 2 by adenosine deaminase, but the subsequent phosphororylytic cleavage to yield hypoxanthine is undetectable. This enzymatic stability toward purine nucleoside phosphorylase is a direct consequence of fluorine's stabilizing effect of the glycosylic bond.

As shown in Figure 1, both compounds compare quite favorably to ddA and ddI after exposure to acidic conditions which destroys the activity of the parent dideoxynucleosides.

The stabilizing effect of the fluorine allowed the synthesis of compounds 4 and 6, which in their unmodified forms (compounds 3 and 5) would be extremely unstable. Unfortunately, these compounds which were considered to be potential leads, failed to display any anti-HIV activity in the ATH8/HIV system.

Synthesis of Potentially CNS-Active Purine Analogues with Increased Liphophilicity (Dr. Driscoll, Dr. Marquez, Dr. Barchi):
The objective of this work is to prepare orally active and acid-stable dideoxypurine nucleosides with partition coefficients in the range log P = - 0.5 to +2.5 in order to maximize blood-brain-barrier penetration. The effect of methyl substitution, expected to increase the lipophilic character of the molecule, was evaluated in a series of analogues of the acid-stable 2′-fluoro-ara-ddA (1, log P = -0.2). An equally important factor was to prevent deamination in all of these compounds to the more hydrophilic hypoxanthine derivatives. Compounds with methyl substituents at positions 2-, 8- and N^6- were prepared and evaluated (7-9). According to known structure-activity data for the enzyme adenosine deaminase, none of these

compounds were expected to behave as good substrates for adenosine deaminase. All of the compounds showed a modest increase in log P value (given under each structure), but only N^6-substitution was compatible with anti-HIV activity. An intermediate compound, the N^6-benzoyl analogue (10), unexpectedly showed significant anti-HIV activity in the ATH8/HIV system.

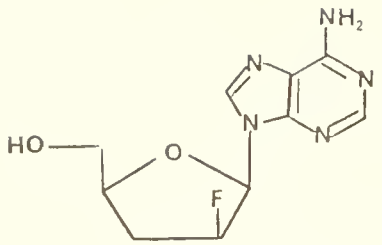
Synthesis of Ring-Enlarged Analogues of Oxetanocin (Dr. Marquez, Dr. Driscoll, Dr. Tseng, Dr. Wysocki):
Oxetanocin (11) is a fermentation product discovered to have anti-HIV activity in the ATH8/HIV system. The preparation of compounds 12 and 13, considered to be ring-enlarged analogues of oxetanocin, demonstrated that the unique four-membered oxetan ring of oxetanocin is not necessary for biological activity. However, the relative orientation of the hydroxymethyl group appears to be critical since only compound 12 showed activity comparable to that of oxetanocin. Compound 12 is considered to be a lead and efforts directed toward the synthesis of a more extensive series are in progress.

Publications:

1. Marquez VE. The Design, Synthesis, and Antiviral Activity of Nucleoside and Nucleotide Analogues. In ACS Symposium Series. Antiviral Agents, Martin JC, ed, in press.

2. Marquez VE. Effect of Fluorine Substitution on the Anti-HIV Activity of Dideoxynucleosides. In NIDA Research Monographs, in press.

3. Driscoll JS, Marquez VE, Kim C-H, Kelley JA. US Patent 4,788,181: 5-substituted-2′,3′-dideoxycytidine compounds with anti-HTLV-III activity, November 29, 1988.



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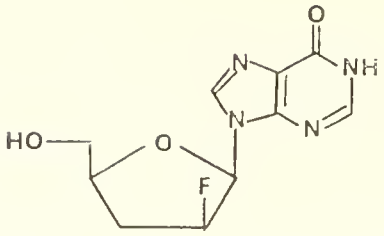

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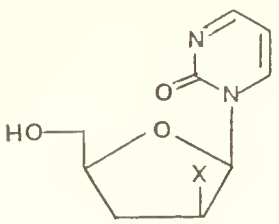
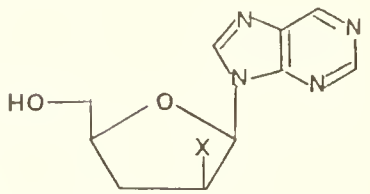


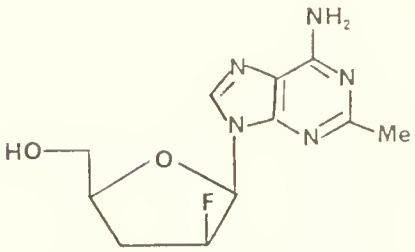
3, X = H

4, X = F

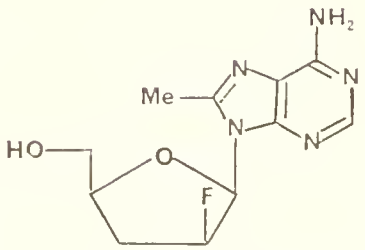


5, X = H

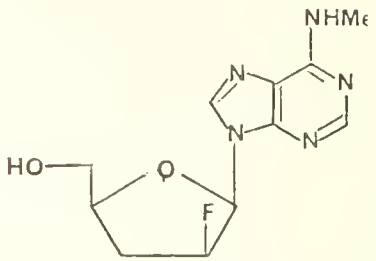
6, X = F



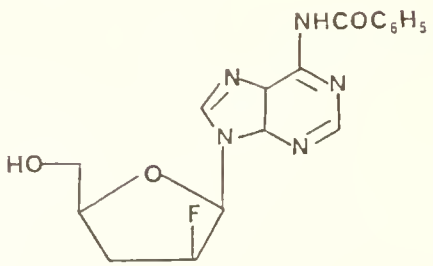
7 (log P = 0.131)



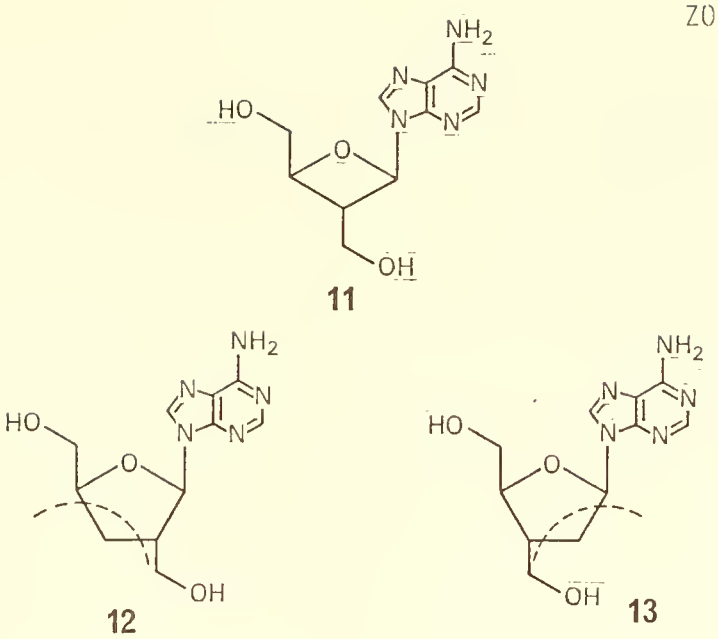
8 (log P = 0.204)



9 (log P = 0.285)



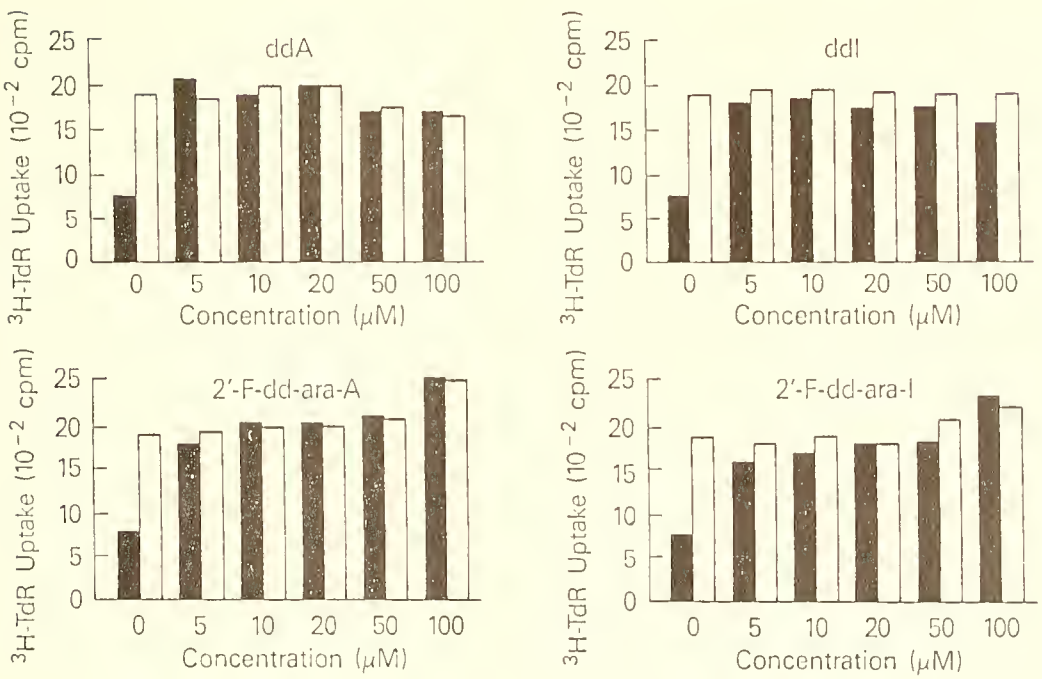
10 (log P = 1.17)



Structures of Oxetanocin and Ring-Expanded Analogues.

FIGURE 1

Protection of ATH-8 Cells from the Cytopatogenic Effects of HIV



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06174-04 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cyclopentenyl Nucleoside Isosteres as Potential Antitumor and Antiviral Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor E. Marquez	Research Chemist	LMC, NCI
Others: John S. Driscoll	Chief	LMC, NCI
Richard R. Copp	IRTA Fellow	LMC, NCI
Michael Bodenteich	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Biochemical Pharmacology, DTP, DCT, NCI.
Southern Research Institute, Birmingham, Alabama.
Katholieke Universiteit, Leuven, Belgium.

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.50	2.25	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This work continues to generate a series of new carbocyclic cyclopentenyl (CPE) nucleoside isosteres that are being studied as antitumor or antiviral agents.

The synthesis of the 3-deaza-CPE nucleoside analogue of the antitumor agent 3-deazauridine was accomplished. Through a similar methodology, the syntheses of 3-deaza-CPE-cytosine and 5-aza-CPE-cytosine were also realized.

A total synthesis of racemic neplanocin F (CPF-A), the allylic rearranged isomer of the potent antitumor and antiviral carbocyclic nucleoside, neplanocin A (CPE-A), was accomplished in 13 steps. Syntheses of other CPF-nucleosides is in progress.

A complete study on the antiviral activity of 3-deazaneplanocin A identified this compound as having very potent and selective in vivo antiviral activity against vaccinia and vesicular stomatitis virus.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: The objective of this work is the systematic evaluation of different structural modifications of cyclopentenyl carbocyclic nucleosides in terms of their antitumor and antiviral properties. These compounds are structurally related to the naturally occurring antibiotic neplanocin A.

Major Findings:

CPE-pyrimidine analogues. (Dr. Marquez, Dr. Copp):
Multistep syntheses of the 3-deaza-cytosine- (3-DA-CPE-C, 1), the 3-deaza-uracil- (3-DA-CPE-U, 2), and the 5-aza-cytosine- (5-A-CPE-C, 3) cyclopentenyl carbocycles were completed. The interesting carbocyclic C-nucleoside 4 was also obtained as a by-product from this work. These syntheses are currently being repeated in order to secure adequate amounts for biological evaluation. Structural and pharmacological considerations regarding the antitumor agents 3-deazauridine, 5-azacytidine and cyclopentenyl cytosine (CPE-C, 5), led to the design and synthesis of the above target structures.

The synthesis of the active metabolite (CPE-CTP, 6) of the antitumor and antiviral agent CPE-C was completed. It was characterized by FAB MS spectrometry and by direct HPLC comparison with the enzymatically generated anabolite. Kinetic inhibition studies with the target enzyme, cytidine triphosphate synthetase, are in progress (Dr. Kang, LBP, NCI).

A synthetic procedure that optimizes the synthesis of isocarbodine (7), a compound used as an internal standard in the analytical protocol for the determination of CPE-C in biological fluids, was developed.

CPE-purine analogues (Dr. Marquez, Dr. Bodenteich): A complete in vitro antiviral evaluation of 3-deazaneplanocin A (8), revealed potent and selective antiviral activity against parainfluenza, vesicular stomatitis, vaccinia, and rotavirus. Excellent antiviral activity was confirmed in vivo in mice infected with vaccinia virus (Dr. Shannon, Southern Research Institute) and in newborn mice infected with vesicular stomatitis virus (Dr. De Clercq, Katholieke Univ., Belgium). In vitro, 3-deazaneplanocin A inhibited rotavirus replication at 0.04 μg/ml, while not being toxic to concentrations of 400 μg/ml. The selectivity index of 10^4 is the highest ever reported for a rotavirus inhibitor.

The total synthesis of racemic neplanocin F (9), an isomer of neplanocin A, was accomplished in 13 steps. The spectral characteristics of this compound matched exactly those of an authentic sample of the naturally occurring enantiomeric product. Samples of neplanocin F have been submitted for biological evaluation.

Publications:

1. Marquez VE, Lim M-I, Treanor SP, Plowman J, Priest MA, Markovac A, Khan, MS, Kaskar B, Driscoll J. Cyclopentenylcytosine. A Carbocyclic Nucleoside with Antitumor and Antiviral Properties. J Med Chem 1988; 31:1687-1694.

2. Marquez VE, Lim M-I, Tseng CKH, Markovac A, Priest MA, Khan SM, Kaskar, B. Total Synthesis of Neplanocin A. J Org Chem 1988; 53: 5709-5714.

3. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim H-Y, Marquez VE, Johns DG.: Cyclopentenylcytosine Triphosphate. Formation and Inhibition of CTP synthetase. J Biol Chem 1989; 264: 713-718.

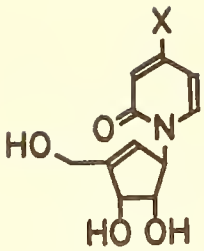
4. Tseng CKH, Marquez VE, Fuller RW, Goldstein BM, Haines DR, McPherson H, Parsons JL, Shannon WM, Arnett G, Hollingshead M, Driscoll JS. Synthesis of 3-Deazaneplanocin A: A powerful Inhibitor of S-Adenosyl Homocysteine Hydrolase with Potent and Selective In Vitro and In Vivo Antiviral Activities. J Med Chem, in press.

5. De Clercq E, Cools M, Balzarini J, Marquez VE, Borcherding DR, Borchardt RT, Drach JC, Kitaoka S, Konno T. Broad-Spectrum Antiviral Activity of Neplanocin A, 3-Deazaneplanocin A and 5′-Nor Derivatives Thereof. Antimicrob Ag Chemother, in press.

6. Driscoll JS, Marquez VE, Plowman J. Cyclopentenyl Cytosine (CPE-C). A Carbocyclic Nucleoside with Antitumor and Antiviral Properties. Nucleosides Nucleotides, in press.

7. Marquez VE, Lim M-I, Khan MS, Kaskar B. (4R,5R)-(-)-3-Benzyloxymethyl-4,5-O-isopropylidene-2-cyclopentenone. An Optically Active α,β-Unsaturated Cyclopentenone for the Synthesis of Neplanocin A and Other Cyclopentene Carbocyclic Nucleosides. In Townsend, L. B. and Tipson, R. S. (Eds.). Nucleic Acid Chemistry. Improved and New Synthetic Procedures Methods and Techniques Part 3, in press.

8. Marquez VE, Lim M-I, Markovac A, Priest MA. (-)-Neplanocin A. In Townsend LB and Tipson RS (Eds). Nucleic Acid Chemistry. Improved and New Synthetic Procedures Methods and Techniques. Part 3, in press.



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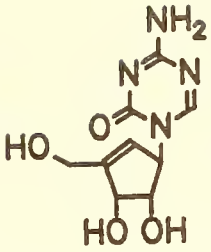

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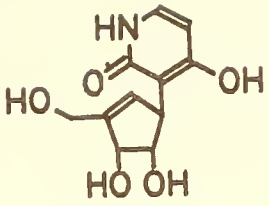

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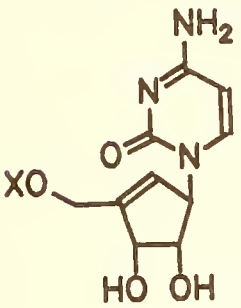
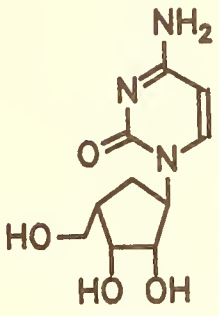


5, X =H



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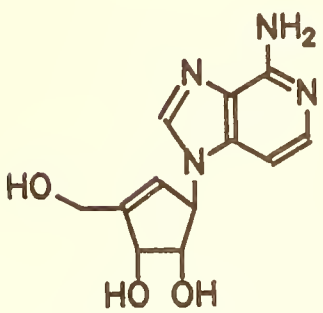
6, X = P(O)(OH)–O–P(O)(OH)–O–P(O)$(OH)_2$



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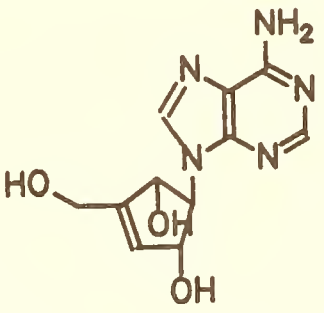


9

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06175-04 LMC

PERIOD COVERED

October 1, 1988, September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthesis and Properties of Oligonucleotides containing 5-azacytosine Residues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

Victor E. Marquez		Research Chemist	LMC, NCI
Others:	Estela Alvarez	IRTA Fellow	LMC, NCI
	Amanda Goddard	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

University of Vermont, Burlington, Vermont

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Manual synthesis of dimers and trimers containing the 5,6-dihydro-5-azacytosine (DHAC) base (e.g. DHACpT and ApDHACpT) have been synthesized. The conversion of the DHAC moiety into the aromatic 5-azacytosine ring (AC) has been accomplished in these compounds. Syntheses of larger oligonucleotides (26-mers) containing the DHAC moiety at specific sites of the sequence have been synthesized in the automated DNA synthesizer by employing a DHAC phosphoramidite reagent developed last year. HPLC methods for the purification of these oligonucleotides are in progress. Biological evaluation of these oligonucleotides is pending.

GPO 914-918

Project Description:

Objectives: The main objective of this work is to develop techniques for the incorporation of 5-azacytosine (AC) and its reduced analogue, 5,6-dihydro-5-azacytosine (DHAC) into specific sites of a synthetic oligonucleotide. The resulting modified oligonucleotides will be studied as specific DNA methylase inhibitors and as probes to study the relationship between DNA methylation and gene expression.

Major Findings:

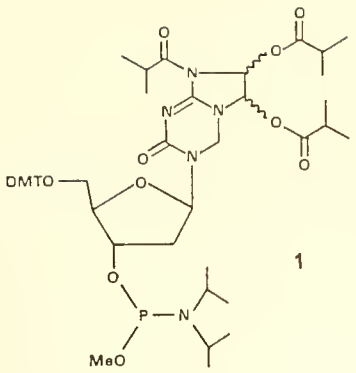
Oxidation of DHAC to AC in a DHACpT dimer (Dr. Marquez, Dr. Goddard): The optimization of the reaction conditions to perform this oxidation required significant amounts of dimer 2. This dimer was initially prepared through the coupling of the phosphoramidite reagent 1 (developed last year in our laboratory) and 3′-O-acetyl-thymidine. Since the conservation of the phosphoramidite 1 was necessary for the automated synthesis of larger oligonucleotides, an alternative route for the synthesis of dimer 2 was developed from more accessible reagents (Scheme 1).

Synthesis of trimers of general formula ApDHACpT and TpDHACpT (Dr. Marquez, Dr. Alvarez): Knowledge of the mechanism of cleavage of DNA at the site of AC incorporation is very important. In order to understand the possible relationship of this instability to the mechanism of inhibition of DNA methylase, synthetic trimers containing the modified base in the middle have been designed. The stability of these AC and DHAC probes will be studied under alkaline conditions with the intention of isolating (by HPLC) and characterizing (NMR, MS) the various fragments. The generation of abasic sites as precursors to the actual cleavage of the phosphodiester bond will be investigated. Initial attempts to make these trimers from dimer 7 failed. Formation of a O^5 -C^6 cyclonucleoside structure in the AC ring after the removal of the dimethoxytrityl group, was responsible for barring the reaction of this intermediate with phosphoramidite reagents. Recently, we have succeeded in the synthesis of trimer ApDHACpT (8) from protected dimer 2. Oxidation of DHAC in this trimer is expected to proceed without difficulty.

Synthesis of modified 26-mers recognized as substrates for DNA methylase (Dr. Marquez, Dr. Alvarez): Synthesis of the parent unmodified 26-mer (5′-CCGGCCATTACGGATCCGTCCTGGGC-3′) proceeded well despite problems in the HPLC purification. This is a highly CG rich oligo which additionally has a great tendency to form hairpin structures. DHAC-modified 26-mers, in which each of the underlined cytosines has been individually replaced by DHAC, have been synthesized. According to gel electrophoresis of the ^{32}P-labeled oligos, there appears to be some degree of failure during the coupling of the DHAC phosphoramidite (1). Efforts directed toward the purification (HPLC) of these oligos continue. Oxidation of the DHAC moiety of these oligonucleotides will provide substrates for the DNA-methylase with a built-in "trojan horse" which is expected to cause enzyme inactivation. The relationship between the specific location of the modified bases and the inhibitory properties of these oligos, should be of great value in understanding the mechanism of action of DNA-methylase.

Publication:

1. Goddard AJ, Marquez VE. Synthesis of Oligonucleotides Containing 5,6-Dihydro-5-azacytosine and 5-Azacytosine at Specific CpG Sites. Nucleosides Nucleotides (in press).



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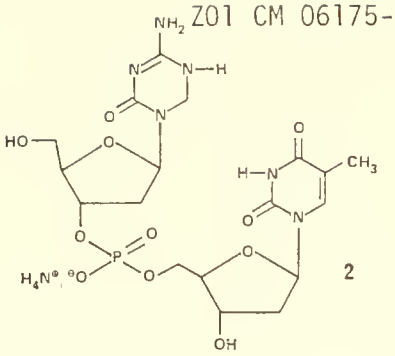

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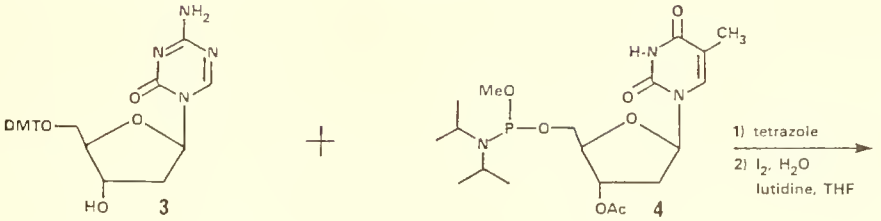
Scheme 1



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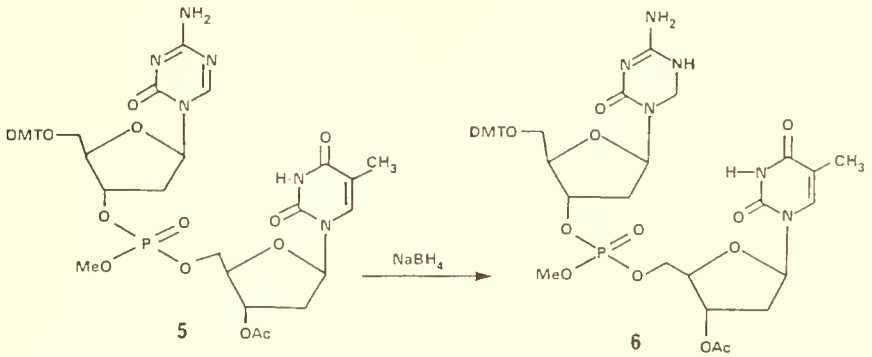

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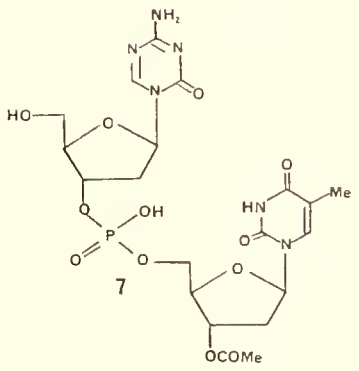

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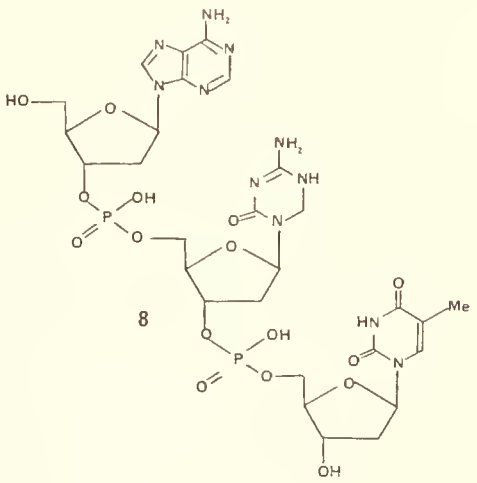


8

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06176-04 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989.

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enzyme Inhibitors as Potential Anticancer and Antiviral Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor E. Marquez		Research Chemist	LMC, NCI
Others:	John S. Driscoll	Chief	LMC, NCI
	Kelley Teng	Visiting Fellow	LMC, NCI
	Joseph J. Barchi	Senior Staff Fellow	LMC, NCI

COOPERATING UNITS *(if any)* Laboratory of Biochemical Pharmacology, DTP, DCT, NCI; Indiana University, Bloomington, Indiana; Laboratory of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI; University of North Carolina, Chapel Hill, North Carolina; Drug Synthesis and Chemistry Branch, DTP, DCT, NCI

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Several compounds which function as rigid analogs of diacylglycerol (DAG), the natural agonist of protein kinase-C, have been synthesized and studied in competitive binding assays with phorbol esters. A clear structure-activity pattern appears to be emerging with a clear potential for developing an effective inhibitor of protein kinase C.

Preliminary findings with certain pyrimidinone nucleosides indicated that the deaminase that works at the nucleotide level (dCMP deaminase) is likewise effectively inhibited by some of these compounds once they are anabolized to the monophosphate level.

New findings relative to the mechanism of inhibition of cytidine deaminase (CDA) identified several pyrimidinone nucleosides synthesized earlier in this laboratory, as the most potent inhibitors of this enzyme with K_i values in the 10^{-12} M range. These compounds also provided some new information regarding the enzymatic mechanism of CDA.

Investigation of mechanism of action of the enzymatically stable phosphonate analogues of TAD (thiazole adenine dinucleotide) and its selenium-containing isostere continues. These compounds behave as inhibitors of inosine monophosphate dehydrogenase.

PHS 6040 (Rev 1/84)

GPO 914-918

Project Description:

Objective:

The objective of this project is to design mechanism-based inhibitors of various enzymes of interest in antitumor or antiviral chemotherapy. Our current approach is based on the construction of a modified substrate which will either inactivate the enzyme, bind tighter to its active site, or resist enzymatic degradation.

Major Findings:

Dinucleotide Analogues of NAD as IMP Dehydrogenase Inhibitors (Dr. Marquez): Larger quantities of both beta-methylene TAD (1) and SAD (2) were prepared through a DTP contractor following our synthetic procedures. The activity of the beta-methylene TAD analogue against L1210 cells resistant to tiazofurin was confirmed (IC_{50} = 25 μM). However, the beta-methylene SAD analogue, expected to be 10-fold more potent than 1, was inactive at 20 μM against both sensitive and resistant L1210 cells. Repetition of these results is in progress as well as evaluation of these phosphonate analogues against the target enzyme, inosine monophosphate dehydrogenase (Dr. David Cooney, LBP, NCI and Dr. H. Jayram, Indiana U.).

Synthesis of Pyrimidine-2-one Ribonucleosides as Transition State Analogues for Cytidine Deaminase (Dr. Marquez): Four compounds (3-6), previously synthesized in our laboratory, were used in the investigation of the mechanism of inhibition of cytidine deaminase. Several studies, including difference UV spectroscopy of the inhibitors with cytidine deaminase, demonstrated that compounds 3 and 4 become the most powerful inhibitors ever reported for this enzyme (K_i values in the 10^{-12} M range) after hydration at the active site. The equilibrium constant for the hydration of 3 (zebularine) was estimated to be 4.7×10^{-6} and the apparent K_i measured for bacterial cytidine deaminase was 2.5×10^{-7} M; this gives a true K_i of 1.17×10^{-12} M for the intermediate 7 which binds seven orders of magnitude tighter to the enzyme that the substrate cytidine. The fluoro compound 4 has been co-crystallized with the enzyme for X-ray studies (Betts, L. et al. J. Biol. Chem. 1989 264: 6737-6740).

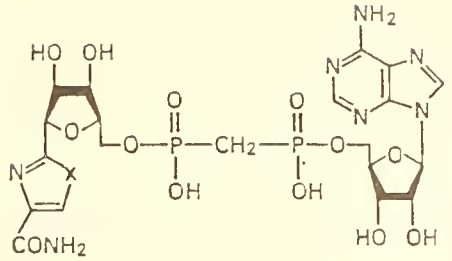
Synthesis of Pyrimidine-2-one Ribosides as Inhibitors of Cytidylate Deaminase (Dr. Marquez, Dr. Barchi, Dr. Cooney, Dr. Driscoll): Preliminary investigations indicate that the significant elevation of dCTP levels observed after treatment of cells in culture with zebularine (3), appear to be due to inhibition of dCMP to dUMP conversion by dCMP deaminase. Since zebularine monophosphate did not prove to be a very potent inhibitor of dCMP deaminase (IC_{50} = 7.4 μM) it is possible that a different zebularine metabolite may be responsible for such inhibition. The fact that inhibition of ribonucleotide reductase by hydroxyurea abrogates the effect of zebularine on dCTP levels, suggests that 2'-deoxyzebularine monophosphate might be the responsible metabolite for dCMP inhibition. With this information, the stable 2'-fluoro-2'-deoxy-ara-zebularine (8), which can be considered as an isostere of 2'-deoxyzebularine, was synthesized and tested. As anticipated, treatment of cells with this drug also caused elevation of dCTP levels and

its monophosphate derivative proved to be a powerful inhibitor of dCMP deaminase (IC_{50} = 0.15 μM). Preparation of additional amounts of monophosphates of these drugs is in progress to clarify the mode of interaction with dCMP deaminase.

Synthesis of Protein Kinase C Inhibitors (Dr. Marquez, Dr. Teng, Dr. Barchi): Significant progress in the synthesis of structurally rigid analogues of diacylglycerol has been achieved. A comprehensive chemical approach that allows the generation of all of the isomeric 2-deoxypentonolactones from readily available chiral precursors (L-ascorbic acid and D-isoascorbic acid) has been devised. With this approach, several final target compounds (9-12) have been synthesized in addition to compounds 13 and 14 reported last year. Selective methods for the specific myristoylation of primary versus secondary alcohol functions have been investigated, as well as methods to prevent a facile acyl migration that appears to occur readily when the stereochemical disposition of the hydroxyl groups is cis. Despite the limited biological data available, it appears that a selective interaction with protein kinase C (PK-C), sensitive to the structural modifications in the molecule, is taking place. The assay system consists in measuring the specific inhibition of the binding of [^{3}H]phorbol-12,13-dibutyrate in the mouse cytosolic phorbol ester aporeceptor. A general SAR analysis is expected to be completed in the near future. The most potent compound prepared to date is 10 with an IC_{50} of 8 μg/ml.

Publication:

1. Goldstein BM, Bell JE, Marquez VE, Robins RK. Dehydrogenase Binding by the Cofactor Analogue TAD. In Molecular Structure. Chemical Reactivity and Biological Activity. International Union of Crystallography Crystallographic Symposia No 2. Stezowski JJ, Huang J-L, Shao M-C. eds. Oxford Univ. Press. New York 1988; pp 163-168.



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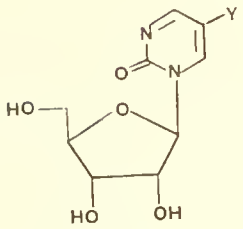

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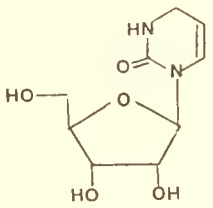
4, Y = F



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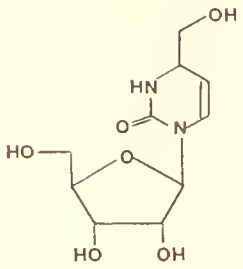

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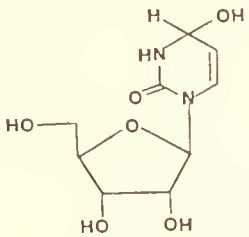

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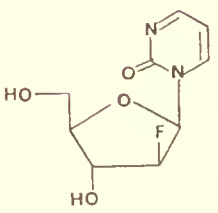

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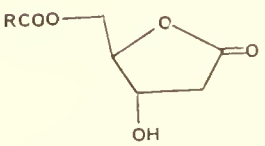

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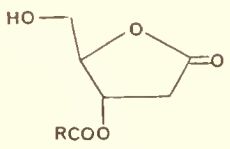

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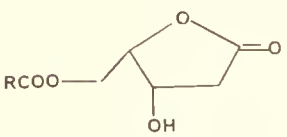

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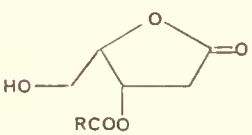

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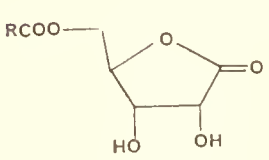

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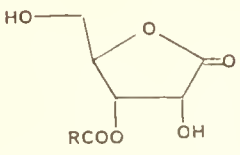

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 03581-20 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Analytical Chemistry of New Anticancer Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James A. Kelley		Research Chemist	LMC, NCI
Others:	John S. Driscoll	Chief	LMC, NCI
	Jeri S. Roth	Chemist	LMC, NCI
	Harry Ford, Jr.	Biotechnology Fellow	LMC, NCI
	Lajos Hegedus	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)* Laboratory of Biochemical Pharmacology, DTP, DCT; Clinical Pharmacology Branch, COP, DCT; Toxicology Branch, DTP, DCT; Pediatric Oncology Branch, COP, DCT; Investigational Drug Branch, CTEP, DCT; Department of Medical Oncology, Walter Reed Army Medical Center.

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.5	1.9	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is the research and development of analytical methods which are used to: (1) establish the structure and purity of new antitumor agents and their metabolites, (2) determine physical and chemical properties of new anticancer drugs, (3) quantitate drugs and their metabolites in biological samples to elucidate pharmacology and to determine pharmacokinetics, and (4) study reaction mechanisms of potentially useful synthetic transformations. Mass spectrometry, gas chromatography and high-performance liquid chromatography, either alone or in combination, are emphasized techniques. Compounds of current interest are cytidine analogs, modified nucleosides, oligonucleotides and differentiating agents.

Project Description:

General Objectives:

The objective of this project is the research and development of analytical techniques for establishing the structure and purity of new anticancer drug candidates, determining their important physical and chemical properties, elucidating structures of metabolites of new antitumor agents, measuring these drugs and their metabolites in biological samples, and studying synthetically useful reaction mechanisms. Collaborative preclinical and clinical studies are conducted to determine in vivo disposition, metabolism, excretion and pharmacokinetics of these new agents. Gas Chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS) and the combination of these techniques are the emphasized methods. Other analytical methods such as NMR, UV and IR spectroscopy are also employed.

Specific Objectives:

1. Plasma pharmacokinetics of arabinosyl-5-azacytosine (Ara-AC) in an adult Phase I clinical trial.

2. Bioavailability, bioequivalence and metabolism of oral dosage forms of hexamethylene bisacetamide (HMBA) in an adult Phase I clinical trial.

3. Analytical methods development and preclinical pharmacology for cyclopentenyl cytosine (CPE-C) and its metabolites.

Major Findings:

1. Plasma Pharmacokinetics of Arabinosyl-5-azacytosine (Ara-AC) in an Adult Phase I Clinical Trial (Drs. Kelley, Ford, Cowan): Ara-AC (NSC 281272) is a new synthetic nucleoside which combines the structural elements of two established antitumor agents, Ara-C (the arabinose sugar) and 5-AC (the triazine base). This drug has been evaluated as a 72-hr continuous i.v. infusion in 27 adult patients with refractory cancer on a Phase I clinical trial. Plasma, pleural fluid and, in one case, CSF have been obtained from 46 cycles of therapy for Ara-AC analysis by our previously developed HPLC assay. Ara-AC has been measured in the plasma of 13 patients receiving 21 cycles of therapy at doses above 1.25 mg/m^2/hr X 72. Plasma steady-state concentrations (C_{ss}) ranged from 32 ng/ml (0.13 μM) to 137 ng/ml (0.6 μM) and were, for the most part, proportional to dose. For one patient, who received 13 cycles of therapy and had the most extensive blood level study, a good linear relationship of measured C_{ss} with dose was observed (Figure 1). The mean clearance for the 21 cycles of therapy for which Ara-AC could be measured was a relatively rapid 647 $\pm$ 141 ml/min/m^2, which was not significantly different from the clearances observed at higher doses on shorter schedules. This rapid clearance is at least partially attributable to the fact Ara-AC undergoes rapid hydrolytic degradation in vivo.

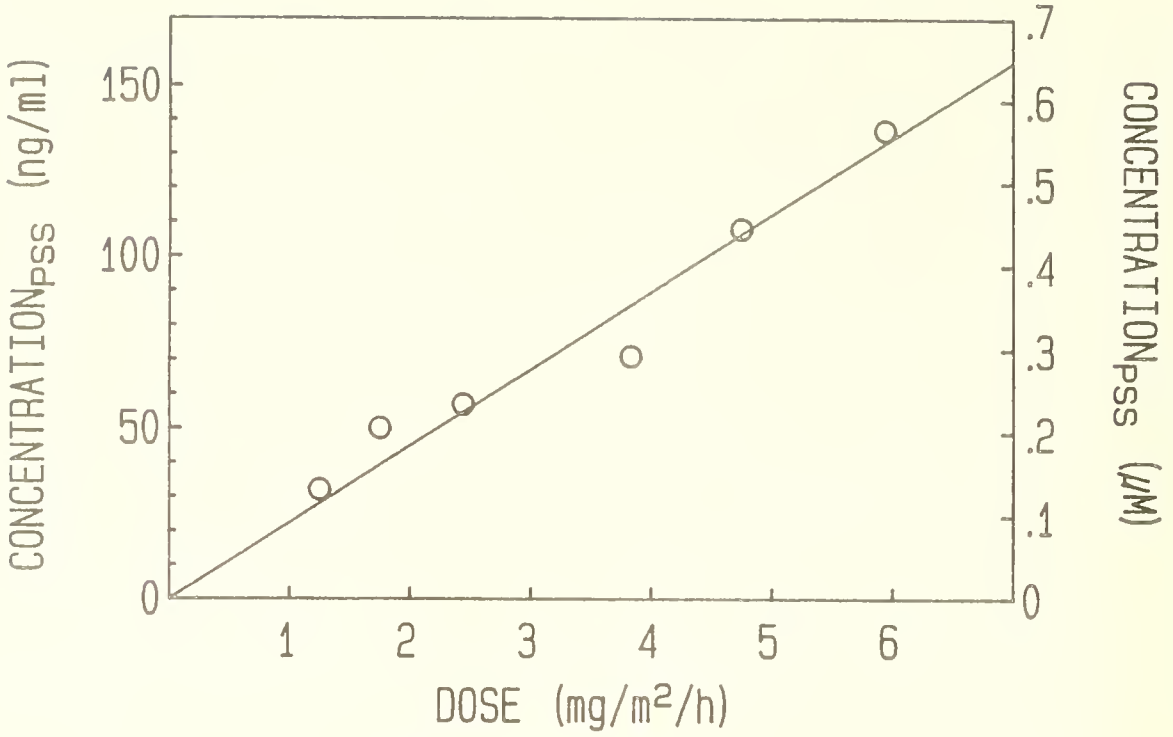
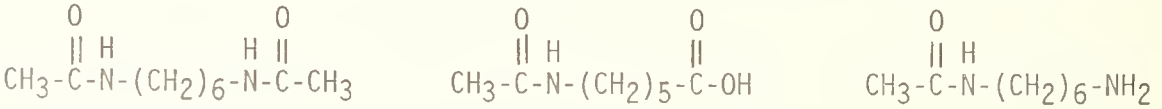


Figure 1. Correlation between Ara-AC plasma concentration at steady-state and dose in a single patient receiving multiple cycles of therapy.

2. Bioavailability, Bioequivalence and Metabolism of Oral Dosage Forms of Hexamethylene Bisacetamide in an Adult Phase I Clinical Trial (Drs. Kelley, Chun, Ward, Ms. Roth): Hexamethylene bisacetamide (HMBA, NSC 95580, 1), a potent in vitro differentiating agent, has been evaluated in a collaborative (NCI and Walter Reed Army Medical Center) Phase I clinical trial to compare a 5 day period of oral administration to a 120-hr continuous i.v. infusion in the same subjects. Since HMBA activity depends on duration of exposure and chronic drug administration may be required, oral dosage forms have been tested because of their better convenience for long-term therapy. Oral bioavailability and dose equivalence for HMBA as a solution administered via nasogastric tube and as a tablet have been evaluated by comparing plasma pharmacokinetics, overall drug exposure (area under the concentration versus time curve or AUC), metabolite plasma concentrations and urinary excretion of HMBA and its metabolites. A previously developed GC method employing selective derivatization and solid phase extraction has been employed to measure simultaneously HMBA and its major acidic (6-acetamidohexanoic acid, 2) and basic (N-acetyl diaminohexane, 3) metabolites in plasma and urine.



For solution HMBA administered via nasogastric tube, absorption from the gastrointestinal tract was rapid with complete bioavailability as determined from plasma AUC measurements. HMBA and metabolite levels in plasma, as well as urinary excretion of 1-3, were also similar to those observed after i.v. administration (Figure 2). In addition, tablet HMBA was found to be bioequivalent to solution HMBA in terms of plasma levels, metabolic pattern and urinary excretion (Table 1).

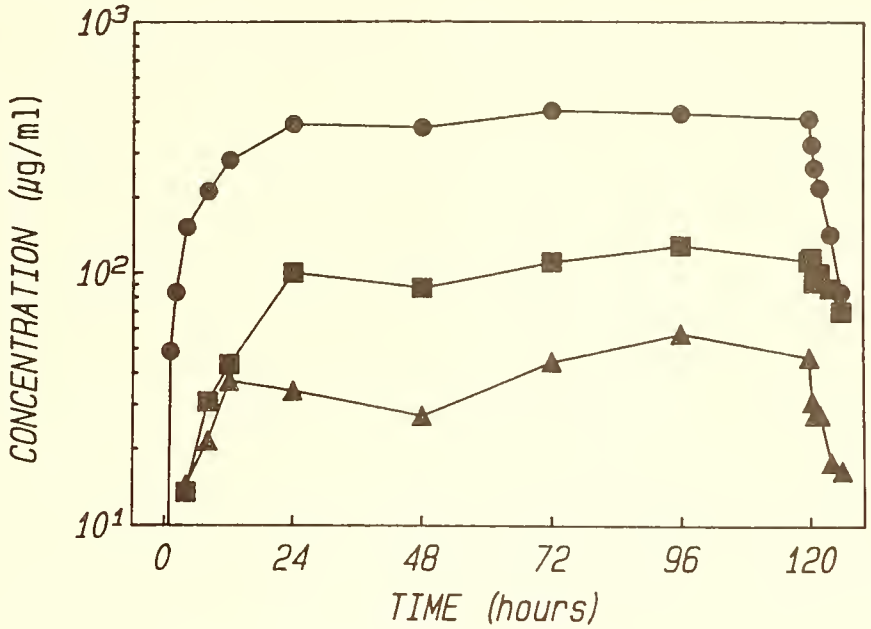


Figure 2. Plasma concentration versus time curve for patient J.K. during and following treatment with 30 gm/m^2/day X 5 HMBA as a continuous infusion. Key: HMBA (●); 6-acetamidohexanoic acid (■); N-acetyl diaminohexane (▲).

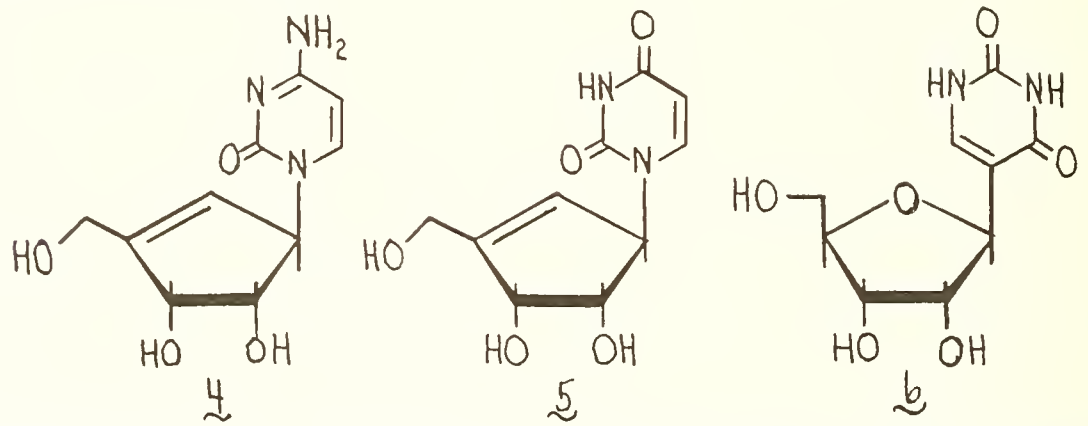
3. Analytical Methods Development and Preclinical Pharmacology for Cyclopentenyl Cytosine (CPE-C):

a. Automated HPLC Assay for Cyclopentenyl Cytosine and its Metabolites (Drs. Kelley, Hegedus, Hartman): Cyclopentenyl cytosine (CPE-C, NSC 375575, 4) is a synthetic cytosine nucleoside containing the same unsaturated sugar as the antineoplastic and antiviral fermentation product neplanocin A. The NCI has designated CPE-C as a candidate for clinical development because of its antitumor activity in several model systems and its potency as an inhibitor of CTP synthetase. An isocratic reverse phase HPLC assay, which is suitable for both preclinical and clinical studies, has been further refined and automated. This assay allows measurement of CPE-C and its deaminated metabolite cyclopentenyl uridine (CPE-U, 5) in plasma, urine and cerebrospinal fluid (CSF) as well as simultaneous detection of endogenous pyrimidine nucleosides such as pseudouridine (6) and cytidine. Pseudouridine is a C-nucleoside which may have some utility as a tumor marker in certain cancers and serve as an additional parameter for measuring the effectiveness of chemotherapy. Sample injection, column switching of the narrow-bore C_{18}, and

Table 1. Comparative Pharmacokinetics and Bioequivalence of Oral HMBA

	J. K.		J. M.		J. P.	
	NG[1]	Tablet	NG	Tablet	NG	Tablet
Dose ($gm/m^2/day$)	30	30	36	36	30	30
Clearance ($ml/min/m^2$)	49	50	61	58	95	111
HMBA steady-state nadir ($\mu g/ml$)	355	378	266	335	104	149
$t_{1/2}$ HMBA (hr)	4.9	3.9	2.7	3.0	1.8	2.6
Mean 6-AHA[2] ($\mu g/ml$)	119	80	62	74	35	25
Mean NADAH[3] ($\mu g/ml$)	48	55	42	29	26	12
Bioequivalence (Tablet/NG)	98%		106%		86%	

[1]HMBA administered as a 5% solution via nasogastric tube
[2]6-Acetamidohexanoic acid
[3]N-Acetyl diaminohexane



data acquisition have been automated to allow unattended operation and high sample throughput.

b. Disposition and Pharmacokinetics of CPE-C in Small Animals (Drs. Kelley, Hegedus, Hartman, Tomaszewski, Zaharko): The bolus dose plasma kinetics of CPE-C were studied in male Sprague-Dawley rats (2 and 20 mg/kg) and in beagle dogs of both sexes (30 and 40 mg/kg). No toxicity was noted at either of the rat doses while the 40 mg/kg dose in dogs was lethal. Plasma elimination in both species was found to be triexponential with the terminal phase having a

Table 2. Pharmacokinetic Parameters for an IV Bolus Dose of CPE-C

Parameter	Beagle Dogs		Sprague-Dawley Rats
	Male	Female	Male
Dose [mg/kg]	30	30	20
(mg/m^2)	(600)	(600)	(120)
3-Compartment Open Model			
AUC [($\mu g/ml$)·min]	8250	5520	9300
$t_{1/2}(\alpha)$ [min]	5.8	1.9	4.6
$t_{1/2}(\beta)$ [hr]	2.14	1.77	0.97
$t_{1/2}(\acute{O})$ [hr]	37.5	21.6	19.2
Cl_{TB} [ml/min/kg]	3.6	5.4	9.0
V_{dss} (ml/kg)	2440	1640	
Trapezoid Rule			
AUC [($\mu g/ml$)·min]	8880	6260	9490
% Extrapolated	9	4	14
Cl_{TB} [ml/min/kg]	3.4	4.8	8.5
Urinary Excretion			
% Dose	49.8	68.5	90.0

very long half-life (Table 2). Consequently, CPE-C plasma levels could still be measured in both dogs and rats at 48 hr after a single bolus dose. Since the limit of quantitation of the analytical methods was 0.1 μM, these concentrations were still in the range of *in vitro* biological activity. Diode array UV scanning of the appropriate HPLC peak was used to confirm the presence of CPE-C at these long time points. Urinary excretion was the major pathway of elimination in both species, with an almost quantitative recovery of unchanged CPE-C after 48 hr in the rat and somewhat less in the dog. No evidence was observed for the catabolism of CPE-C to CPE-U in either species. These data were combined with contractor data from mice to develop a general pharmacokinetic model in an attempt to explain drug toxicity and to predict CPE-C disposition in other animals and in humans. This model incorporates a deep compartment that is small but has high concentrations of CPE-C that are slowly released to generate the terminal phase of elimination. The concentration profile of this compartment may represent the uptake and slow release of CPE-C as its phosphates and may be more predictive of toxicity than measurements of parent drug.

c. Disposition, Metabolism and Pharmacokinetics of CPE-C in Non-human Primates (Drs. Kelley, Hegedus, Blaney, Balis, Heideman, Poplack): A study has been initiated to investigate CPE-C disposition, metabolism and

pharmacokinetics in rhesus monkeys. Parent drug was rapidly eliminated from plasma in an apparent biphasic manner (terminal half-life = 14.4 hr) after an i.v. bolus dose of 5 mg/kg (100 mg/m^2) to a male monkey. Toxicity was marked but manageable with anorexia, RBC hemolysis and a decrease in WBC for at least 14 days. CPE-C could be measured in plasma for 6 hr and in the CSF shortly after drug administration, but central nervous system penetration was poor (CSF/plasma $\leq$ 7%). Less than 20% of the administered dose was excreted as parent drug in the urine. In contrast to mice, rats and dogs, CPE-U was detected in plasma, CSF and urine; and its presence was confirmed by diode array UV scanning of the appropriate HPLC peak. Concentration of this metabolite approached that of CPE-C in plasma and greatly exceeded parent drug in CSF. CPE-U also accounted for the remaining fraction of administered dose in the urine. Further studies are under way to confirm these results and evaluate CPE-C as a substrate for cytidine deaminase.

4. Synthetic and Collaborative Project Support (Drs. Kelley, Ford): Numerous samples which cannot be categorized as coming from any one project area have been analyzed by the appropriate mass spectral and chromatographic techniques on an individual basis. Included in this group are modified nucleosides, neplanocin analogs, carboacyclic nucleosides, dideoxyribose nucleosides, and mono- and oligonucleotides.

Publications:

1. Heideman RL, Roth JS, Ford H Jr., Kinnard RD, Litterst CL, Kelley JA. Reverse phase HPLC determination and murine pharmacokinetics of arabinosyl-5-azacytosine. J Liquid Chromatogr, in press.

2. Heideman RL, Gillespie A, Ford H, Reaman GH, Balis FM, Tan C, Sato J, Ettinger LW, Packer RJ, Poplack DG. Phase I trial and pharmacokinetic evaluation of fazarabine in children. Cancer Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06177-04 LMC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Analytical Chemistry of Anti-AIDS Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James A. Kelley	Research Chemist	LMC, NCI
Others: John. S. Driscoll	Chief	LMC, NCI
Harry Ford, Jr.	Biotechnology Fellow	LMC, NCI

COOPERATING UNITS *(if any)*
Laboratory of Biochemical Pharmacology, DTP, DCT; Clinical Oncology Program, DCT; Digestive Diseases Branch, NIDDK.

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is the research and development of suitable analytical methods to: (1) establish the structure and purity of potential anti-AIDS agents and new antiviral drugs, (2) determine physical and chemical properties, including octanol-water partition coefficients, of these compounds and their metabolites, and (3) measure these drugs and their metabolites in biological samples to elucidate pharmacology and to determine pharmacokinetics. Gas chromatography, high-performance liquid chromatography and mass spectrometry are emphasized techniques. Compounds of current interest are dideoxyadenosine, dideoxyinosine, dideoxyguanosine, 2′,3′-dideoxy-2′-fluoro-β-D-arabinofuranosyl adenine and 2′,3′-dideoxy 2′-fluoro-β-D-arabinofuranosyl hypoxanthine.

PHS 6040 (Rev 1/84) GPO 914-918

Project Description:

General Objective:

The objective of this project is the research and development of suitable analytical methods for establishing the structure and purity of new anti-AIDS drug candidates, determining their important chemical and physical properties, elucidating structures of metabolites of these new agents, measuring these compounds and their metabolites in biological samples, and studying reaction kinetics and mechanisms of synthetically important transformations. High-performance liquid chromatography (HPLC) and mass spectrometry are the preferred techniques. Other analytical methods such as NMR, UV and IR spectroscopy, and ion exchange and affinity chromatography are also employed.

Specific Objectives:

1. Pharmacokinetics of 2′,3′-dideoxypurine nucleosides in a duck model for human hepatitis B.

2. Chemical and physical properties of both base and sugar-modified dideoxynucleosides, with an emphasis on the rapid microscale determination of octanol-water partition coefficients.

Major Findings:

1. Pharmacokinetics of 2′,3′-Dideoxypurine Nucleosides in a Duck Model for Human Hepatitis B (Drs. Ford, Mitsuya, Hoofnagle): Pekin ducks chronically infected with duck hepatitis B virus, a hepadnavirus containing reverse transcriptase, are a model for human hepatitis B, a similar virus which chronically infects approximately 5% of the world's population. Intravenous bolus administration of dideoxynucleosides has been shown to decrease viral replication, as measured by DNA polymerase activity, in this duck model. Analytical methods have been developed and modified to measure selected dideoxypurine nucleosides in the duck in order to define plasma kinetics and correlate blood levels with antiviral activity. A reverse phase HPLC method, based on that developed for measurement of 2′,3′-dideoxyadenosine (ddA), has been modified to allow quantitation of both 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxyguanosine (ddG) in duck plasma. Isolation and concentration from the plasma matrix by solid phase extraction on a C_{18} cartridge followed by isocratic (7% CH_3CN in 0.01 M pH 6.8 phosphate buffer) HPLC analysis with UV detection at the wavelength maxima gives a limit of quantitation of 0.1 μM for each dideoxynucleoside. This HPLC assay was used to determine plasma elimination kinetics following an i.v. bolus dose of each drug in the above Pekin duck model. Unlike ddI and ddG, ddA was unstable in duck plasma at 41.3°C (normal duck body temperature) and was rapidly converted to ddI. Accordingly, ddI was measured following dosing with ddA. All drugs showed biphasic elimination profiles with comparable kinetic parameters.

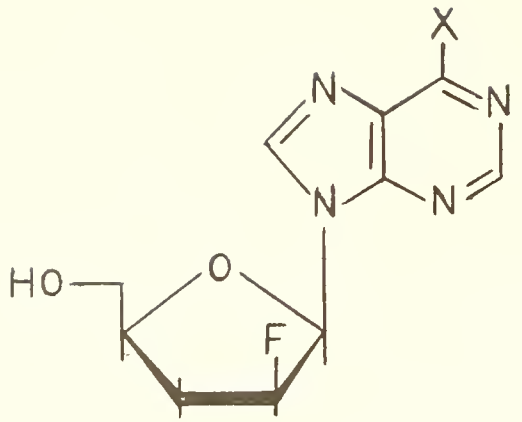
Table 1. Pharmacokinetics of Dideoxypurine Nucleosides in the Pekin Duck

			ddG		
	ddA[1]	ddI	I	II	III
Dose [μg/kg]	814	857	857	857	857
$t_{1/2}(\alpha)$ [min]	2.3	5.7	4.3	3.8	3.6
$t_{1/2}(\beta)$ [min]	36	33	16	19	51
AUC [μg/ml·min]	36.5	29.6	65.0	13.5	45.2
CL_{TB}[2] [(ml/min)/kg]	22	29	13	64	19
MRT[3] [min]	44	32	13	18	55
V_{dss} [ml/kg]	971	929	174	1152	1034

[1]Measured as ddI.
[2]Total body clearance.
[3]Mean residence time.

2. Chemical and Physical Properties of Base and Sugar-Modified Dideoxynucleosides (Drs. Kelley, Ford, Marquez): Further studies were carried out to characterize chemically and enzymatically the acid-stable series of purine dideoxynucleosides incorporating a flourine atom in the 2′- "up" position of the sugar. Both 2′,3′-dideoxy-2′-fluoro-β-D-arabinosyl adenine (1, 2′-F-dd-ara-A) and 2′,3′-dideoxy-2′-fluoro-β-D-arabinofuranosyl hypoxanthine (2, 2′-F-dd-ara-I) were found to be acid-stable and quantitatively recoverable under conditions in which ddA and ddI were completely decomposed. Various adenine nucleosides were also evaluated as substrates for adenosine deaminase (ADA). When equivalent amounts of ADA were used at room temperature, the conversion of ddA to ddI was almost instantaneous, 1 was deaminated to 2 rapidly, but more slowly than ddA, and N^6-methyl-2′,3′-dideoxy-2′-fluoro-β-D-arabinofuranosyl adenine (3, N^6-Me-2′-F-dd-ara-A) did not undergo observable conversion.

Microscale methods which would allow a rapid determination of lipophilicity and octanol-water partition coefficients are currently being investigated for both pyrimidine and purine dideoxynucleosides which are candidate anti-AIDS agents. The ability of HIV to infect and damage the central nervous system (CNS) requires therapeutic agents that can cross the blood-brain barrier. Thus, partition coefficients are important for an initial assessment of which of a series of synthesized 2′-F-dideoxypurine derivatives possessing appropriate lipohilicity to enter the CNS. Among the methods being evaluated are an isocratic HPLC system using a C_8 reverse phase column,



1 X = NH_2

2 X = OH

3 X = $NHCH_3$

a gradient HPLC system employing ion-pairing on a C_8 reverse phase column at pH 3.2, and a shake-flask method which utilizes a micro extraction apparatus. Current work has focussed on defining the advantages and limitations of each system by using model compounds and selected unknowns. Table 2 presents some results of preliminary studies.

Table 2. A Comparison of Partition Coefficients Determined by HPLC

	Log P			
2'-F-dd-ara-Nucleoside	Isocratic	Gradient	Micro Shake-Flask	DTP Contractor
Inosine (2)	-0.716			-1.18
Purine	-0.187			
Adenosine (1)	STD	STD	-0.173	-0.097
2-Methyl-adenosine	0.131	0.427	0.155	
8-Methyl-adenosine	0.204	0.335	0.065	
N^6-Methyl adenosine (3)	0.285	0.276	0.314	
2,N^6-Dimethyl-adenosine	0.618	0.804	0.619	
N^6-Benzoyl-adenosine	1.17	1.27		
AZT	0.146	-0.109	0.045	0.038

Publications:

1. Collins JM, Klecker RW Jr., Kelley JA, Roth JS, McCully CL, Balis FM Poplack DG. Pyrimidine dideoxyribonucleosides: selectivity of penetration into cerebrospinal fluid. J Pharmacol Exp Ther 1988;245:466-70.

2. Kassianides C, Hoofnagle JH, Miller RH, Doo E, Ford H, Broder S, Mitsuya H. Inhibition of duck hepatitis virus replication by 2′,3′-dideoxycytidine. a potent inhibitor of reverse transcriptase. J Gastroenterology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06178-04 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Applications of New Mass Spectral Techniques

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James A. Kelley	Research Chemist	LMC, NCI
Others: Harry Ford, Jr.	Biotechnology Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is research on and development of new mass spectral techniques in order to provide new and/or more rapid solutions to problems involving (1) chemical structure determination, (2) complex mixture analysis and (3) measurement of trace components in biological systems. The scope and utility of these mass spectral methods are determined, and a comparison to other types of analyses, both new and established, is carried out. Fast atom bombardment mass spectrometry and combined liquid chromatography-mass spectrometry are the techniques of current interest. Fast atom bombardment mass spectrometry in both the positive and negative ion mode continues to be applied for the rapid structure determination of nucleosides, nucleotides and natural products. A microscale desalting procedure has been shown to be suitable for the semipreparative scale purification of synthetic nucleosides and nucleotides.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

General Objective:

The objective of this project is the development and application of new mass spectral techniques for the rapid analysis of complex mixtures, measurement of trace components in biological systems and chemical structure determination. Fast atom bombardment mass spectrometry and combined liquid chromatography-mass spectrometry are the new techniques of current interest. The advantages and limitations of these new methods to already established techniques is also an area of concern.

Specific Objectives:

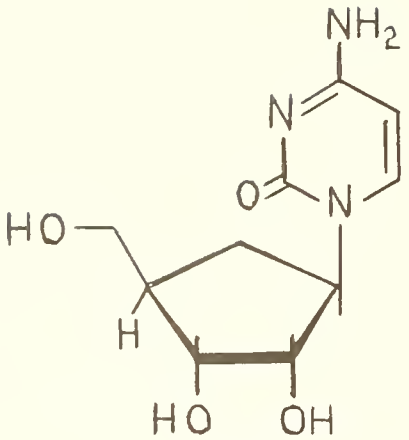
1. Rapid structural analysis of nucleosides, nucleotides and natural products by fast atom bombardment mass spectrometry.

2. Evaluation and application of a microscale minicolumn desalting procedure for semipreparative purification of synthetic samples.

Major Findings:

1. Rapid Structural Analysis of Nucleosides, Nucleotides and Natural Products by Fast Atom Bombardment (FAB) Mass Spectrometry (Drs. Kelley, Fales): The LMC continues to conduct an extensive program in the synthesis of nucleosides and nucleotides of novel structure. Rapid and simple methods employing FAB mass spectrometry are employed to characterize these nucleic acid constituents without derivatization. Determination of elemental compositions from accurate mass measurement of FAB molecular ions has been utilized for structure confirmation of sugar-modified nucleosides. An investigation is currently in progress on the suitability of various FAB liquid matrices for compound classes such as nucleosides, nucleotides and organic peroxide natural products.

2. Semipreparative Minicolumn Purification (Drs. Kelley, Ford): The suitability of minicolumn desalting for the semipreparative purification of nucleosides and nucleotides was investigated. Cytidine was chosen as a model compound because it is the least retained of the pyrimidine ribosides upon reverse phase chromatography and therefore the most difficult case. A mandatory test of compound purity for the synthetic chemist is a satisfactory elemental analysis. Since a commercial carbon, hydrogen and nitrogen analysis usually requires 5-10 milligrams of compound, desalting an equimolar mixture of cytidine and NaCl at the 40 μmole level was evaluated. Greater than 96% of the salt could be removed in one chromatography cycle. After overnight drying at 100°C to completely remove water, elemental analysis results were within 0.08% of calculated values for all three elements. Semipreparative scale desalting of AMP (30 μmole, 10 mg) was also effective, being faster yet just as efficient as gel filtration or semipreparative HPLC. This method was successfully applied to the milligram scale purification of a dihydro-5-azacytosine-thymine nucleotide dimer and to the semipreparative separation of carbodine (1) and isocarbodine (2) for additional chemical and biological characterization.



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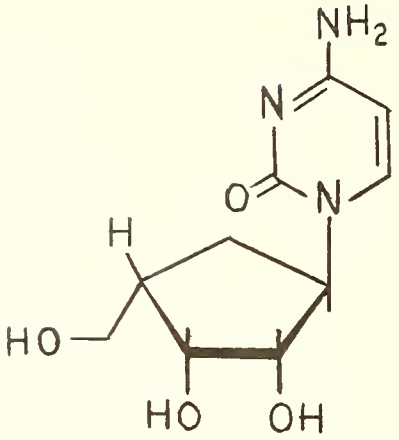

2

Publications:

1. Moon D-C, Kelley JA. A simple desalting procedure for fast atom bombardment mass spectrometry. Biomed Environ Mass Spectrom 1988;17:229-37.

2. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim H-Y, Marquez VE, Johns DG. Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase. J Biol Chem 1989;264:713-18.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06194-01 LMC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Modelling and Drug Design by Computer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

	G. W. A. Milne	Chemist	LMC, NCI
Other:	John S. Driscoll	Chief	LMC, NCI
	Victor E. Marquez	Research Chemist	LMC, NCI
	Peter P. Roller	Research Chemist	LMC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A molecular modelling capability has been established in the Laboratory of Medicinal Chemistry. The utility of this technique in drug design is being explored and its application to various specific problems is being pursued.

Protein Kinase C. Two distinct types of competitive inhibitor to phorbol appear to exist and the molecular characteristics of each of these has been established. This supports the design of new inhibitors and leads to a more precise definition of the active site or sites in the enzyme.

Reverse Transcriptase Inhibitors. The method is able, on the basis of molecular architecture, to rank candidate structures for synthesis according to probability of activity.

HIV Protease. The recently completed structure of this protein from HIV has been modelled and work aimed at understanding its proteolytic action and the design of potential inhibitors is in progress.

Three-Dimensional Data Base. The need for a database of 3-dimensional atomic coordinate data has become very clear and work has begun to generate such a database from the NCI DIS file of 400,000 chemicals. Access to such a file will relieve the current dependency upon x-ray diffraction data as a basis for molecular modelling.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

General Objective:

The objective of this project is to to provide, by means of molecular modelling by computer, a physico-chemical rationale for the design of chemical molecules with various biological and chemotherapeutic activities.

Specific Objectives:

1. Design of inhibitors of protein kinase C.

2. Design of compounds with anti-HIV activity.

3. Study of the active site of HIV protease.

MAJOR FINDINGS

Design of inhibitors of protein kinase C

It has been known for some time that 1,2-diacyl glycerols can compete with phorbol for the active site of protein kinase C. The ribose derivative 2-deoxy-3-myristoylribonolactone and its three stereoisomers have been modelled and the models compared to that of phorbol. The two trans-isomers of the lactone have geometric features that are very similar to those of phorbol leading to the selection of these two compounds as potential phorbol inhibitors.

The allylic alcohol moeity of phorbol seems also to be able to lodge in a receptor and other, quite different allylic alcohols have been found to inhibit phorbol binding to protein kinase C. The pharmacophore involved has been characterized geometrically and a search of the x-ray crystal database produced some 90 other compounds, including some of LMC's own carbocyclic nucleosides, that possess the pharmacophore.

Design of compounds with anti-HIV activity

Molecular models have been developed of a large number of dideoxynucleosides, particularly in the adenosine series. This has permitted the definition, with increasing precision, of several criteria which must be met by a compound if it is to inhibit reverse transcriptase and thus demonstrate anti-HIV activity. Important amongst these criteria are the molecular dimensions of the dideoxynucleoside structure and a number of new compounds which meet these criteria are currently being synthesized.

Study of the active site of HIV protease

The human immunodeficiency virus (HIV) contains a proteolytic enzyme of 99 amino acid residues. The structure of this protease has been established by x-ray crystallography and a 3-dimensional model of the molecule has been

developed. Study of this model has been undertaken with a view to understanding the mode of action of the enzyme and, possibly, design and development of inhibitors to it.

PUBLICATIONS

1. Milne GWA. Use of a CENTRUM Workstation in a Drug Design Laboratory. Proceedings of the London Annual Online Meeting. 1988; 12: 189-201 .

2. Milne GWA. Molecular Modelling in Drug Design. Proceedings of the Montreux 1989 International Chemical Information Conference, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06195-01 LMC

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Molecular Recognition Phenomena and Development of Inhibitors to HIV1 Protease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Peter P. Roller		Research Chemist	LMC, NCI
Other:	John S. Driscoll	Chief	LMC, NCI
	George W. A. Milne	Research Chemist	LMC, NCI
	Motoyoshi Nomizu	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)*: Laboratory of Experimental Carcinogenesis; DCE, NCI

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Efforts are focused in the design, synthesis and evaluation of polypeptides that are expected to act as effective inhibitors of key biochemical processes in cell proliferation and in retroviral invasion. In particular, inhibitors to the HIV-1 virally encoded aspartate protease have been designed and their synthesis initiated. This enzyme is required for processing of the gag-pol polyprotein, hence for maturation of the virus. Design strategy made use of the "sense/anti-sense" concept of recognition phenomenon. Design was also improved by using a computer-aided hydropathic complementary score difference minimization program. It is expected that these inhibitory peptides will have high affinities for the catalytic site of the viral enzyme and also for the intermolecular contact sites of its homodimeric active form. Synthesis of peptides is in progress and peptide-peptide affinity studies, HIV protease inhibition assays, and in vitro viral assays will be carried out in the near future.

Project Description:

Objective:

1. Design, synthesis and evaluation of effective inhibitors to the virally encoded (HIV-1) aspartate protease. 2. Evaluation of the "sense/anti-sense" concept of molecular recognition phenomenon as an approach to the design of inhibitory agents and of affector molecules of receptor substrate complexes.

The peptide chemistry group was recently established and the experimental work has just begun.

Methods Used:

(1) Analysis of the 3-dimensional structure of proteins, enzymes, etc. (2) Application of the hydrophobicity/hydrophylicity properties of constituent amino acids to the design of recognition molecules. (3) Computer-assisted generation of anti-sense peptides by minimization of the hydropathic complementarity score differences. (4) Solid phase peptide synthesis and purification methods. (5) Affinity constant determinations by analytical affinity chromatography. (6) Molecular weight and structure determinations by fast atom bombardment mass spectrometry. (7) Conformational studies by circular dichroism spectropolarimetry. (8) HPLC based enzyme inhibition assays.

Approach and Major Findings:

The virally encoded gag-pol polyprotein of HIV-1 contains a 99 residue segment that acts as a virus specific protease at seven cleavage sites of the polyprotein itself. This enzyme activity is required for viral maturation. The recently disclosed X-ray structure of the viral protease (Navia et al, 1989) allows for accurate examination of the molecular geometry for inhibitor design.

Our approach allows for exploring the development of three types of inhibitors, all based on using the "sense/anti-sense peptide" concept initially proposed by Blalock in 1984. However, the preferred structures for synthesis are those that are designed using the computer optimization method developed recently in our joint work with Omichinski and Fassina (1988-89). The latter approach consistently provides peptides with better affinities, when compared to the DNA-based anti-sense peptides. The three approaches are:

1. Ten residue peptides encompassing residues 21-30, were designed that are expected to bind with high affinities to the catalytic site of the enzyme, thus preventing enzyme action on the viral polyprotein substrate. Synthesis of these peptides is in progress.

2. The active enzyme is a homodimer. On examining the three-dimensional structure, it is possible to discern contact site segments of the constituent monomers. Two of these regions encompass residues 45-55 and 85-94. Peptides designed to bind to these regions should prevent dimerization, and therefore

enzyme activation. We have designed several 10-residue peptides as initial candidates for synthesis.

3. A third approach involves preparing peptides that would bind with high affinity to the known cleavage sites in the gag/pol polyprotein, thus preventing enzyme action. We demonstrated in preliminary experiments with the putative ATP binding region of the c-raf oncogenic protein that this latest approach has some promise.

Evaluation of the peptides will be done at three levels: (1) the demonstration that the designed peptides possess binding affinity to the constituent segments will be done using analytical affinity chromatography; (2) enzyme inhibition assays will be carried out with purified HIV1 protease; and (3) in vitro assays with various HIV infected cell lines will be performed. Based on the test results, the promising peptides will be further improved by consideration of molecular geometries, hydropathic properties and molecular modeling in order to generate inhibitors with better affinities, cell permeabilities and resistance to proteolytic enzymes. Our preliminary results indicate that anti-sense peptides synthesized with D-amino acids show good affinities for target sites.

Publications:

1. Han K-H, Niu, C-H, Roller PP and Ferretti JA. Conformation of the second disulfide loop in human transforming growth factor α studied by two-dimensional NMR spectroscopy. Biopolymers. 1988; 27: 923-937.

2. Fales HM, Blum MS, Southwick EK, Williams DL, Roller PP and Don AW. Structure and synthesis of tetrasubstituted pyrazines in ants in the genus **Mesoponera**. Tetrahedron. 1988; 44: 5045-5050.

3. Niu, C-H, Han K-H and Roller PP. Comparison of the conformation and GTP hydrolysing ability of N-terminal ras p21 protein segments. Biochem Biophys Res Commun. 1989; 160: 282-288.

4. Benjamin T, Niu C-H, Parmellee DC, Huggett Ac, Yu B, Roller PP Thorgeirsson SS. Direct N-terminal sequence analysis of rat liver plasma membrane glycoproteins separated by two dimensional polyacrylamide gel electrophoresis. Electrophoresis, in press.

5. Fassina G, Roller PP, Olson AD, Thorgeirsson SS and Omichinski JG. Recognition properties of peptides hydropathically complementary to residues 356-375 of the c-raf protein. J Biol Chem, in press.

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR PHARMACOLOGY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1988 to September 30, 1989

The major goal of the Laboratory of Molecular Pharmacology (LMP) is to obtain basic knowledge that could be applicable to the development of new strategies for the selective killing of human tumor cell types.

DNA Topoisomerases as Targets of Anticancer Drugs

DNA topoisomerases are receiving a great deal of attention in several laboratories as potential new chemotherapeutic targets and are an ongoing focus of work in the LMP. This area of investigation grew out of studies conducted in the LMP about 10 years ago using the DNA filter elution methodology which had been developed in the Laboratory. This work had shown that several clinically active anticancer drugs, including doxorubicin, amsacrine and other DNA intercalative drugs, affect DNA in a manner that was suggestive of an effect on a DNA topoisomerase. Subsequent work in the LMP further developed and strengthened this hypothesis which was finally verified by work carried out both at Johns Hopkins University and in the LMP. In 1984, the Johns Hopkins studies showed that the enzyme topoisomerase II that they had isolated and characterized was affected in the expected manner by the drugs, a finding that greatly simulated the field because it made possible studies in purified systems. Recent LMP studies have aimed to correlate the drug effects in purified systems with effects on intact cells or isolated cell nuclei. In addition to the DNA intercalative anticancer drugs, it has been found that the etoposides (VP-16 and VM-26) are specific inhibitors of topoisomerase II, and that camptothecin is a specific inhibitor of topoisomerase I; these drugs feature prominently in current work in the LMP.

During the current year, we completed a structure-activity study of 35 camptothecin derivatives in which the effects on purified topoisomerase I were found to be closely correlated with previously reported activity against L1210 leukemia in mice. This provides strong evidence that effects on topoisomerase I are the cause of the antitumor activity. In addition, the structure-activity relation for topoisomerase I inhibition indicated that the drug binds to an asymmetric site on the enzyme or enzyme-DNA complex and provided clues to the geometry of the drug-binding site. The results show where the drug molecule can be changed so as to add substituents that would increase the water solubility or otherwise alter the pharmacologic properties of the drug, without loss of activity on topoisomerase I. Since lack of solubility in water is a major limitation in the potential effectiveness of camptothecin as an anticancer drug, special attention was given to this aspect of the problem, and a derivative was identified that has increased solubility without loss of potency in topoisomerase I assays. The work has also indicated how further chemical modification could combine increased solubility with increased potency.

We have investigated the mechanism by which drug-stabilized topoisomerase-DNA complexes lead to cell death. We found that inhibition of DNA synthesis by aphidicolin or hydroxyurea can protect cells essentially completely to the cell killing effect of camptothecin, even though the number of camptothecin-induced protein-associated strand breaks is unchanged. For the case of the topoisomerase II inhibitors, amsacrine and etoposide, the DNA synthesis inhibitors produced partial protection. The results suggest that the cell killing is not due merely to the presence of trapped topoisomerase-DNA complexes, but rather is the result of an interaction of these complexes with replication forks.

Several laboratories are trying to determine the mechanisms by which cells can become resistant to topoisomerase II inhibitors, and several diverse mechanisms involving alterations in topoisomerases have emerged. In the resistant cell line that we have been studying, a unique mechanism has emerged in which the resistance to topoisomerase II inhibitors is associated with the appearance of an altered topoisomerase I. Our current evidence suggests that what is involved is an alteration in topoisomerase phosphorylation by a protein kinase. We are investigating the possibility that the resistance in these cells may be due primarily in an altered protein kinase activity.

Our results suggest that multidrug resistance often entails two seemingly independent factors: increased P170 glycoprotein which enhances drug efflux and topoisomerase II alterations which affect drug responses independent of the efflux mechanism.

We are investigating the DNA sequence patterns found at the sites of drug-induced stabilization of topoisomerase I and II complexes. We find that the sites of stabilization depend in part on the identity of nucleotides in the immediate vicinity of the cleavage sites. The preferred sequence patterns depends on the drug used.

DNA Sequence Selective Alkylating Agents:

Since nitrogen mustards such as cyclophosphamide and melphalan continue to play a major role in cancer chemotherapy, despite their nonspecific chemical reactivities, we are investigating how the selectivity of the reaction of this class of drugs might be increased so as to perhaps greatly increase their clinical utility. We have focussed on the reactions at DNA guanine-N7 positions, since these are the major reaction sites and the most likely primary sites of drug action. We are investigating the dependence of reaction selectivity on the DNA sequence pattern in the neighborhood of the reaction site. Our previous studies had revealed major differences in the intensities of the reactions of nitrogen mustards dependent upon the DNA sequence around the reaction site, and had indicated that the origin of the reaction selectivity could be understood in terms of the calculated electrostatic potential at the reaction sites. We had also found that, among 7 nitrogen mustards selected on the basis of clinical usefulness or potential interest, 5 exhibited similar patterns of DNA sequence selectivity, while 2 showed unique and unusual sequence selectivities. This demonstrated that the sequence selectivity could be altered by manipulating the chemical structure of the nitrogen mustard, thereby providing new opportunities for drug development. The 2 unusual drugs were uracil mustard and quinacrine mustard, and in each case it was possible to achieve at least a partial understanding of the

origin of the unique selectivities in structural terms. We have now surveyed 35 additional nitrogen mustards from the drug inventory of the Development Therapeutics Program, and have found 2 new cases of unique reactivities which we are currently investigating, Because of the differences between DNA in free solution and DNA in nuclear chromatin, we investigated the effects of ionic strength, divalent cations, polyamines and DNA intercalators; we found that, although the overall reaction intensities are altered, the DNA sequence selectivities are remarkably resistant to these changes. This suggests that the findings with DNA in free solution may be relevant to DNA in the cell. . .To test this further, we have reconstituted nucleosomes on a DNA fragment and so far have found little change in selectivity.

We have further examined the pharmacologic differences among nitrogen mustards by determining the kinetics of DNA crosslink formation and repair in cells. Major differences were observed among the commonly used nitrogen mustards. The results suggested that it may be possible to extend classical pharmacokinetic models to intracellular compartments which would include DNA crosslinks, the likely effective pharmacologic targets, as a pharmacokinetic compartment.

DNA Damage and Repair in Specific Regions of the Genome

We have been studying the preferential repair of DNA lesions in transcriptionally active, as opposed to inactive, regions of the genome in mammalian cells. Preferential repair appears to be a crucial mechanism for the ability of cells to survive DNA damage. A survey of human cells derived from patients having various known genetic disorders of DNA repair revealed deficient preferential repair of UV lesions in Cockaynes syndrome and in Gardiner's syndrome. This is the first time that human cells have been found that are incapable of preferential repair.

We have developed new methodologies that allow these studies to be carried out on a variety of DNA lesions, and we have verified that both nitrogen mustard adducts and cisplatin adducts are repaired preferentially in the active dihydrofolate reductase gene, as compared to inactive regions flanking this gene.

We have been investigating the functioning of the chromosome cycle in normal and tumor cells in proliferating and quiescent states. We have continued to focus on the H2A histone family, which we previously identified as having several interesting characteristics. Unique among the four nucleosomal histone families, the H2A family contains species which have been conserved as separate discernable entities during evolution. There are three such species: H2A.1, H2A.Z, and H2A.X, the latter two of which were originally identified in this laboratory. We have now cloned cDNAs or genes for all three species. We have cloned the human gene for H2A.Z, the most dissimilar of these three species, and we are investigating the control of the expression of this gene in proliferating and quiescent cells. We have also isolated and sequenced the human cDNA for H2A.X. This previously unsequenced protein was thought to contain a unique carboxy-terminal region. Quite unexpectedly, the protein sequence of this carboxy-terminal region was found to be highly homologous to that of the major H2As of yeast and other lower eukaryotes. These findings indicate that the histone H2A family has unique roles in the functioning of the chromosome cycle.

The H2A.Z gene is of particular interest, because it is expressed in cycling, but not in quiescent cells. Its regulation thus may be similar to that of the myc oncogene product. We have sequenced the H2A.Z gene of human, cow and rat and found it to contain four introns and to be flanked upstream by two Alu repeat sequences. The level of H2A.Z mRNA was found to decrease by a factor of 20 when proliferating cells become quiescent.

We have continued to investigate the dependence of the interactions between histone and DNA on the presence or absence of DNA replication. We have developed methods to identify histones not bound to chromatin, and are investigating their characteristics. We have found evidence that these "soluble" histones are present in complexes, and we are now investigating the interactions of these complexes with DNA. These interactions between histone and DNA are important during replication of chromatin, during the functioning of chromatin in quiescent cells, and in gene transcription.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06140-13 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Regulation of Histone Biosynthesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William Bonner	Sr. Investigator	LMPH NCI
Others:	Nancy Touchette	Sr. Staff Fellow	LMPH NCI
	Concepcion Muneses	Chemist	LMPH NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	1.0	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Our major objective is to elucidate how histones package and interact with DNA to form chromatin, during the presence of DNA replication as in S phase cells and during the absence of DNA replication as in G1 and quiescent or GO cells.

1. We have developed methodology which allows us to study soluble histone not bound to chromatin and to perform kinetic studies on the alterations in the level of soluble histone in different cell growth states and during changes in the rates of protein or DNA synthesis. Results of these kinetic studies show that there are multiple kinetic components (published).

2. Current biochemical characterization of soluble histone suggests that it is bound in a negatively charged complex that sediments at 8-9s.

3. Current studies to reconstruct chromatin assembly in vitro using cellular extracts containing soluble histone, have demonstrated the transfer of some soluble histone to added DNA, but much work remains in characterizing this system.

The significance of this project lies in the characterization of the cellular interactions between histone and DNA, interactions involved in basic cellular processes such as replication and transcription.

GPO 914-918

Project Description

Introduction:

Histone protein synthesis and DNA synthesis are closely co-ordinated; inhibition of one leads to inhibition of the other. However, when DNA synthesis is inhibited, histone mRNA levels fall and when protein synthesis is inhibited, histone mRNA levels rise. Recently we published a model which suggested that inhibition of protein synthesis led to the inhibition of DNA synthesis by the depletion of histone from the soluble cellular fraction. In contrast to earlier models, this one viewed the resulting stabilization of histone mRNA as part of the same process that led to its destabilization when DNA synthesis was inhibited.

We have developed methodology which allows us to study the flux of histone through the soluble cellular fraction. These methods can also be applied to cell cultures in which either protein or DNA synthesis has been inhibited as well as to cell cultures in different states of growth (G1, GO, and S).

Objectives:

1) To elucidate the molecular mechanisms governing the interactions between histones and DNA in the presence and absence of chromatin biosynthesis during the cellular states of proliferation and nonproliferation.

2) To biochemically characterize the chromatin or cellular components that may be involved in these regulatory mechanisms.

3) To reconstruct chromatin assembly in vitro using cellular extracts.

Methods:

(1) Discontinuous electrophoretic separation of histones including direct loading of histone extracts and two dimensional electrophoresis. (Methods developed in this laboratory).

(2) Synchronization of cell lines, particularly human Hela cells and Chinese hamster ovary cells for studies on cell cycle.

(3) Maintenance of cells and nuclei in viable non dividing states using modified and defined media.

(4) Biochemical techniques such as sucrose and glycerol gradient centrifugation, isoelectric focusing, agarose gel electrophoresis

Major Findings and Accomplishments:

1. Development of Methodology to Study Soluble Histone

Soluble histone has not been studied because of several technical difficulties. These include the small amount of material, the problems of purifying histone proteins from cytoplasmic supernatants, and the problem of distin-

guishing soluble histone from possibly contaminating chromatin histone. We have adapted our methodology for the analysis of histone variants in chromatin to the analysis of histones in the cytoplasm and have overcome most of these problems. Qualitative modification differences between soluble and chromatin-bound histone control for the problem of contaminating chromatin-bound histone. Thus soluble histone can be confidently analyzed free of contamination from chromatin-bound histone.

2. Investigation of the Soluble Histone Pool

We have used these newly developed methods to study how soluble histone levels are altered when the balance of histone and DNA synthesis is altered. Results published since the last annual report show that when protein synthesis is inhibited, the rate of DNA synthesis falls in a biphasic manner. The initial fall has a half time of about 1 min. When the rate of DNA synthesis has fallen to 25% of the initial rate, the rate then falls more slowly with a half time of about 40 min. The rate of soluble histone depletion similar; the kinetics are also biphasic and the rates are similar to those for the deceleration of DNA synthesis. These results are consistent with our model that DNA synthesis can be limited by the availability of histone.

Conversely, when DNA synthesis is inhibited, the soluble histone level is elevated, with H4 and H3 being elevated more than H2B and H2A. The latter two histones continue to be incorporated into chromatin for sometime in the absence of DNA synthesis. We have also been able to demonstrate that H2B and H2A from prelabeled nuclei can be found in the cytoplasm while H4 and H3 are not. This result suggests that H2B and H2A can exchange between the nucleus and cytoplasm in both directions.

Further experiments with G1 cultures have shown that H4 and H3 are elevated in the cytoplasm, while H2B and H2A are not detectable. This finding suggests that the same mechanism is operating in G1 cells as in S cells with inhibited DNA synthesis. Experiments with G0 cells are in progress.

3. Biochemical Characterization of Soluble Histone

The purpose of these experiments is to isolate and characterize factors which interact with soluble histone. Current biochemical characterization of soluble histone suggests that it is bound in a negatively charged complex that sediments at 8-9s. We are currently engaged in further characterization of these particles.

4. Chromatin Assembly in vitro

Using soluble cellular extracts, we are currently engaged in studies to reconstruct the chromatin assembly process in vitro. We are still in the initial stages of these studies, but to date we have demonstrated the transfer of some soluble histone to added DNA.

Significance to Biomedical Research and Program of the Institute:

Chromatin assembly during DNA replication is recognized as a basic cellular process. Histone interaction with non-replicating DNA is also an important cellular process, involved with DNA repair and transcription.

Proposed Course:

1. To characterize soluble histone levels and to compare the predictions of the published model to the behavior of soluble histone.

3. To isolate and characterize factors which interact with soluble histone using typical biochemical techniques and assays developed during the kinetic studies.

4. To investigate the biochemical aspects of the interactions of histone with DNA in the presence and absence of DNA replication.

5. To reconstruct chromatin assembly in vitro using cellular histone complexes.

Publications:

Bonner WM, Wu RS, Panusz HT, Muneses C. Kinetics of accumulation and depletion of soluble newly synthesized histone in the reciprocal regulation of histone and DNA synthesis, Biochemistry 1988;27:6542-50.

Bonner WM, Wu RS, Panusz HT, Muneses C. Qualitative and kinetic characterization of soluble histone pools: linkage between protein and DNA synthesis during the cell cycle. In Kelly, T, Stillman B, eds. Cancer cells, eukaryotic DNA replication, vol 6. New York:Cold Spring Harbor Laboratory, 1988;269-78.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06150-08 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Protein-Associated DNA Breaks as Indicator of Topoisomerase Inhibition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Yves Pommier	Visiting Associate	LMPH NCI
Others:	Donna Kerrigan	Chemist	LMPH NCI
	Christine Jaxel	Visiting Fellow	LMPH NCI
	Kurt W. Kohn	Lab Chief	LMPH NCI

COOPERATING UNITS *(if any)*
Cleveland Clinics Foundation, Cleveland, OH 44106 (Dr. Ram Ganapathi)
Laboratory of Molecular Carcinogenesis, NCI (Dr. Kenneth H. Kraemer)

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

DNA topoisomerases are major targets for cancer chemotherapy. Topoisomerase I is concentrated in nucleoli and topoisomerase II is a major component of the nuclear scaffold for interphase nuclei and of the metaphase chromosome scaffold. Topoisomerase II activity is required for chromosome segregation during mitosis and both enzymes are involved in DNA metabolism. Camptothecin inhibits topoisomerase I, and DNA intercalators (amsacrine, anthracyclines) and demethylepipodophyllotoxins (VP-16 & VM-26) inhibit topoisomerase II. Enzyme inhibition results from enzyme-linked DNA breaks, which are believed to be the initial cytotoxic lesions of the drugs. However, the breaks reverse quickly upon drug removal. This prompted us to determine the cytotoxic lesion(s) induced by the topoisomerase-linked DNA breaks. In the case of topoisomerase II inhibitors, we had previously found that sister chromatid exchanges and mutations could represent such cytotoxic lesions (Pommier et al, Cancer Res 1987;48:512-6). After further work, we now have found that the topoisomerase I-DNA complexes induced by camptothecin lead to irreversible alterations of DNA replication complexes and cell death. We have also determined that topoisomerase II-mediated DNA breaks are concentrated in replicating DNA and that enzyme linkage to the replicons occurs after these replicons had reached full size. We have confirmed our previous finding that pleiotropic resistant cell lines selected by exposure to adriamycin are cross-resistant to other topoisomerase II inhibitors and have reduced topoisomerase II-mediated DNA cleavage. Thus, topoisomerase II alterations may play a crucial role in the mechanism of pleiotropic resistance. Finally, we have found that topoisomerase I is increased in a cancer prone genetic disease, Bloom syndrome.

PHS 6040 (Rev 1/84) GPO 914-918

1. Study the cellular events leading to cell death upon exposure to topoisomerase inhibitors.

2. Determine the involvement of topoisomerase II in the pleiotropic resistance of cells selected by exposure to adriamycin.

3. Isolate topoisomerases from resistant cells and study their regulation by protein kinases.

4. Determine the involvement of topoisomerases in cancer prone genetic diseases.

Methods:

1. Standard cell culture and clonogenic assays.

2. Alkaline elution to quantify DNA breaks and DNA-protein crosslinks.

3. Preparation of nuclear extracts; topoisomerase purification by anion exchange chromatography; and immunoblotting with topoisomerase antibodies.

4. DNA topoisomerase assays with SV40 DNA: filter binding and agarose gel electrophoresis and autoradiography of ^{32}P-end labeled DNA fragments (new DNA cleavage assay).

Major Findings:

1. Cytotoxic Mechanism(s) of Topoisomerase Inhibitors in Chinese Hamster DC3F cells:

We have investigated the possibility that topoisomerase-DNA complexes could be lethal by interfering with DNA replication. First, we found that 30 min treatments with a single topoisomerase inhibitor inhibited DNA synthesis by 50% and that this inhibition could be complete only by combining both topoisomerase II and I inhibitors. DNA synthesis inhibition was almost irreversible after drug removal although the topoisomerase-mediated DNA breaks reversed within 30 min. Next, we found that short pretreatments (5 min) with aphidicolin, a specific inhibitor of DNA polymerase, completely abolished the cytotoxicity of camptothecin and only partially that of the topoisomerase II inhibitors, etoposide and amsacrine (30 min drug treatments). Concurrently, aphidicolin had no effect upon drug-induced topoisomerase-linked DNA breaks. These results show that camptothecin is cytotoxic in S-phase but only in the presence of active replication, which suggests that the topoisomerase I-DNA complexes induced by camptothecin may form steric blocks irreversibly altering replication complexes and leading to cell death.

2. Localization of Drug-induced Topoisomerase II-DNA Complexes in Replicons:

Replicating DNA was separated from bulk DNA by differential labeling with ^{3}H- and ^{14}C-thymidine followed by alkaline elution and pH step elution. The topoisomerase II-mediated DNA break frequency was found to 2-3-fold greater

in replicating DNA. Replicons were covalently linked to topoisomerase II in the presence of VP-16 or amsacrine only when they had reached approximately full size, while shorter replicons were not linked to the enzyme. These results indicate that topoisomerase II is located at some distance from replication forks and that DNA attachment seems to occur only after completion of the replicons. This finding is in agreement with the established finding that topoisomerase II is located at the base of chromatin loops.

3. Topoisomerase II Modifications as a Mechanism of Resistance to Doxorubicin (ADR) in Pleiotropic Resistant Cells:

We had previously reported that doxorubicin-resistant breast cancer (MCF 7/ADR) cells formed markedly less topoisomerase II-mediated DNA breaks upon exposure to VP-16 and that topoisomerase II activity was reduced in nuclear extracts from MCF 7/ADR cells (Sinha et al, Cancer Res 1988;48:5096-100). We have now extended our study to mouse leukemia L1210 cells made resistant to ADR. These cells were found to have increased P170 glycoprotein, to be cross-resistant to vincristine, and thus to exhibit a typical pleiotropic resistant phenotype. However, they were also cross-resistant to other topoisomerase II inhibitors (VP-16 & amsacrine), and nuclear extracts were resistant to the induction of DNA breaks by the drugs. A remarkable characteristics of these cells is that resistance to induction of topoisomerase II-mediated DNA breaks was correlated with resistance to ADR and other topoisomerase II inhibitors, while reduced drug uptake could not account for the degree of cellular resistance. These results indicate that multi-drug resistance is multifactorial and that resistance to topoisomerase II-mediated DNA cleavage could represent a major resistance determinant.

4. A 68 kDa Topoisomerase I and Phosphorylation of Topoisomerases by Protein Kinase C in Chinese Hamster Cells Resistant to 9-Hydroxyellipticinium (DC3F/9-OHE Cells):

We had previously reported the presence of an unusual topoisomerase activity with high DNA-linking properties in these cells. We have now purified the enzyme by anion exchange chromatography. The 68 kDa enzyme is a topoisomerase I except for its lower molecular weight, its ATP-stimulated decatenation, DNA linking and cleavage activities. Antibodies made against the purified 68 kDa topoisomerase I did not cross react with antibodies against the 100 kDa topoisomerase I and vice versa. The effects of ATP were due to enzyme phosphorylation by a protein kinase C gamma which co-purified with the 68 kDa topoisomerase. Dephosphorylation of the 68 and 100 kDa topoisomerases I inactivated both enzymes, which could be reactivated by partially purified protein kinase C. Enzyme inhibition by camptothecin was also found to depend on the phosphorylation state of topoisomerases. These results indicate that the resistant cells contained increased protein kinase C gamma, which co-purified with a 68 kDa topoisomerase I. The role of these alterations in the cellular resistance remains to be determined.

5. Increased Topoisomerase I Activity in Transformed Bloom Syndrome Cells:

Bloom syndrome (BS) is a rare autosomal disease characterized by ultraviolet light hypersensitivity and the occurrence of lethal cancers early in life.

The presence of an unusually high frequency of sister chromatid exchanges in cells from BS patients led us to investigate the presence of topoisomerase alterations in these cells. Two cell lines of transformed BS fibroblasts and lymphoblasts were compared to similarly transformed normal cell lines. Bromodeoxyuridine produced DNA breaks in all 4 cell lines. These breaks were not protein-linked and were alkali labile indicating that they did not result from topoisomerase cleavage. Irradiation with 313 nm light induced more protein-linked breaks in BS than in normal cells. However, we found no evidence for their induction by topoisomerases. Topoisomerase II activities were similar in normal and BS cells. Unexpectedly, topoisomerase I activity as measured by camptothecin-induced DNA breaks and cellular content of topoisomerase I as measured by immunoblotting with antibodies from a patient with sceloroderma, were approximately 2-fold greater in BS than in normal cells. The relationship between this increased topoisomerase I activity and the reported defect of DNA ligase I in BS cells remains to be investigated.

Proposed Course:

1. Develop and study the cellular effects of new camptothecin analogs which could be developed as anticancer agents.

2. Determine whether topoisomerase II alterations generally contribute to drug resistance in adriamycin-resistant cell lines exhibiting the pleiotropic phenotype.

3. Identify the cellular lesions responsible for cell death after exposure to topoisomerase inhibitors.

4. Investigate the role of protein kinases in catalytic activity and drug sensitivity of topoisomerases.

Publications:

Pommier Y, Kohn KW. Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins. In: Glazer I ed. Developments in Cancer Chemotherapy. Boca Raton: CRC, 1989;175-95.

Sinha B, Haim N, Dusre L, Kerrigan D, Pommier Y. DNA strand breaks produced by etoposide (VP-16,213) in sensitive and resistant human breast tumor cells: implications for the mechanism of action, Cancer Res 1988;48:5096-100.

Pierson V, Pierre A, Pommier Y, Gros P. Production of Protein-associated DNA breaks by 10-[diethylaminopropylamino]-6-methyl-5H-pyrido[3′,4′:4,5]pyrrolo[2,3-g]isoquinoline in cultured L1210 cells and isolated nuclei: comparison with other topoisomerase II inhibitors, Cancer Res 1988;48:1404-09.

Holm C, Covey J, Kerrigan D, Kohn KW, Pommier Y. DNA synthesis inhibition suppresses the cytotoxicity of the topoisomerase I inhibitor, camptothecin, and has limited effects upon topoisomerase II inhibitors, Proceeding of the Second Conference on DNA Topoisomerases as Targets for Cancer Chemotherapy, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06161-06 LMPH

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Topoisomerases as Target of Action of Anticancer Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Yves Pommier	Visiting Associate	LMPH NCI
Others:	Christine Jaxel	Visiting Fellow	LMPH NCI
	Giovanni Capranico	Guest Researcher	LMPH NCI
	Donna Kerrigan	Chemist	LMPH NCI
	Kurt Kohn	Lab Chief	LMPH NCI

COOPERATING UNITS (if any)

University of Texas Health Science Center, Dallas, TX 75235 (Dr. William T. Garrard)

LAB/BRANCH: Laboratory of Molecular Pharmacology, DTP, DCT

SECTION:

INSTITUTE AND LOCATION: NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.9	2.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

DNA cleavage induced by inhibitors of topoisomerases I (camptothecin) and II (anthracyclines, amsacrine, VP-16, VM-26, and ellipticines) can be produced by purified mouse leukemia L1210 topoisomerases in ^{32}P-end labeled DNA fragments. Both purification of topoisomerases and preparation of DNA fragments were performed in the laboratory.

Drug activities and mapping of DNA cleavage sites induced by different inhibitors were investigated in order to study the molecular interactions between drugs and topoisomerase-DNA complexes.

Topoisomerase II activity in reconstituted SV40 chromatin was also investigated. First, we found that SV40 DNA contained a nuclear matrix attachment site (MAR) located in the early transcription region (nucleotides 4071-4377) and that the same region contained the highest density of topoisomerase II cleavage sites. These results demonstrate the existence of a MAR in a viral genome (surprisingly in a coding sequence) and confirm the involvement of topoisomerase II in the nuclear matrix architecture. Secondly, we found that topoisomerase II-mediated DNA cleavage was suppressed in reconstituted nucleosomes, and therefore enzyme activity depends upon chromatin structure.

Objectives:

1. Use purified enzymes and DNA fragments to study drug effects and to select the most active topoisomerase inhibitors that could be studied in cells and animal tumor models.

2. Map topoisomerase II cleavage sites in the SV40 genome in order to sequence the most intense ones and to look for a consensus sequence of DNA cleavage sites.

3. Determine the molecular interactions between drugs and topoisomerase-DNA complexes.

4. Compare drug-induced DNA cleavage in free and nucleosome reconstituted DNA.

Methods:

1. Purification of DNA topoisomerases from mammalian cells in culture by anion exchange chromatography.

2. ^{32}P-end labeling of SV40 DNA fragments and oligonucleotides.

3. DNA sequencing gels and autoradiography to determine the DNA sequence at topoisomerase-induced DNA cleavage sites.

Major Findings:

1. Evidence for a Stereospecific Receptor Site for Camptothecin in Topoisomerase I-DNA Complexes:

Inhibition of purified mammalian topoisomerase I by thirty compounds related to camptothecin was studied. Stereochemistry and the positions of substituents were found to be crucial for topoisomerase I inhibition, indicating that camptothecin interacts with an asymmetric receptor site on the enzyme or enzyme-DNA complex. From the structure-activity relations, the regions of interaction between the camptothecin ring system and the receptor site were inferred. Striking correlations were observed between activity against topoisomerase I and reported activity against murine leukemias, suggesting that topoisomerase I inhibition is responsible for the antitumor activity of camptothecin. In addition, some camptothecin derivatives substituted on the A ring appeared to be more potent than camptothecin: 10- or 11-hydroxycamptothecin, 10,11-methylenedioxycamptothecin, and 9-glycinaminocamptothecin; the latter derivative is water soluble indicating that substitution at the 9-position could be a way to synthesize camptothecin derivatives that could be developed as anticancer agents.

2. Topoisomerase II Cleavage Sites Induced by Adriamycin Derivatives in SV40 DNA; Sequence Localization and Relation to Antitumor Activity:

The compounds included doxorubicin, daunorubicin, two doxorubicin stereoisomers (4'-epimer and ß-anomer), and five chromophore-modified derivatives,

with a wide range of cytotoxic activity and DNA binding affinity. Cleavage of ^{32}P-end-labeled DNA fragments was visualized by autoradiography of agarose and polyacrylamide gels. Structure-activity relationships indicated that alterations in the chromophore structure greatly affected drug action on topoisomerase II. In particular, removal of substituents on position 4 of the D-ring resulted in more active inhibitors with lower DNA binding affinity. The stereochemistry between the sugar and the chromophore was also essential for activity. All the active anthracyclines induced a single region of prominent cleavage in the entire SV40 DNA, which resulted from a cluster of sites between nucleotides 4237 and 4294. DNA cleavage intensity patterns exhibited differences among analogues and were also dependent upon drug concentration. Intensity at a given site depended on both stimulatory and suppressive effects depending upon drug concentration and DNA sequence. A good correlation was found between cytotoxicity and intensity of topoisomerase II-mediated DNA breakage.

3. Nuclear Matrix Attachment Sites and Topoisomerase II Cleavage Sites map to the same region of SV40 DNA:

^{32}P-end labeled DNA fragments were used to map the binding sites of nuclear matrices and topoisomerase II in the entire SV40 genome. Binding of nuclear matrix from mouse cells was determined by a competition assay (Cockerill and Garrard, Cell ...) and found to be limited to a fragment encompassed between nucleotide .... and ..... The same DNA region was found to contain the most intense cleavage site of mouse leukemia topoisomerase II and multiple other strong sites. These results are in agreement with other findings demonstrating that topoisomerase II is a major component of the nuclear matrix. Unexpectedly, they show that the matrix attachment site (MAR) of SV40 is in the large tumor antigen gene, in a region which forms nucleosomes.

4. Nucleosome Formation Suppresses Topoisomerase II-mediated DNA Cleavage Induced by Antitumor Topoisomerase II Inhibitors:

The effect of core histones upon topoisomerase II binding to DNA was investigated by comparing the cleavage sites of purified enzyme induced by four antitumor topoisomerase II inhibitors (teniposide, amsacrine, 5-imino-daunorubicin, and 2-methyl-9-OH-ellipticinium) in purified and in nucleosome reconstituted SV40 DNA. SV40 nucleosomes were reconstituted by incubating [^{32}P]-end labeled linear DNA fragments with an excess of HeLa cell mononucleosomes in 0.7 M NaCl for 20 min at 37^{o}C, and by diluting slowly these mixtures in a step-wise manner to 70 mM NaCl. Chromatin reconstitution was checked by a gel shifting assay and nucleosome positioning by comparing micrococcal nuclease digestion patterns of chromatin with those of naked DNA. Three types of DNA regions were identified: 1) regions with 150 bp segments protected from nuclease digestion separated by sites of enhanced digestion, indicative of fixed nucleosome positioning, 2) regions of global protection without precise nucleosome positioning, and 3) one region of 200-300 bp around the origin of replication which did not seem reconstituted. Drug-induced topoisomerase II cleavage sites were markedly affected by nucleosomes. Most of them were suppressed at sites of nuclease protection and other cleavage sites appeared stronger at sites of nuclease enhancement. These results indicate that DNA bound to nucleosomes is not available for

topoisomerase II cleavage and suggest that core histone binding could regulate topoisomerase II activity.

Proposed Course:

1. Use purified DNA and topoisomerase I to select new, more active, and water-soluble camptothecin derivatives which could be studied further in cellular and animal tumor systems.

2. Analyze the DNA sequence at the major cleavage sites of various topoisomerase II inhibitors in SV40 DNA, and look for consensus sequences as a function of drug structure and concentration.

3. Use oligonucleotides derived from DNA sequence analyses to determine the minimum length of DNA fragments required for topoisomerase I and II cleavage; DNA sequence mutations could then be used to study the DNA sequence requirements for drug-induced DNA cleavage.

4. Oligonucleotides could also be used to determine the molecular interactions of drugs with topoisomerase-DNA complexes. ^{3}H-camptothecin could be used for these studies.

Publications:

Jaxel C, Kohn KW, Pommier Y. Topoisomerase I interaction with SV40 DNA in the presence and absence of camptothecin, Nucleic Acids Res 1988;16:11157-70.

Jaxel C, Kohn KW, Wani MC, Wall ME, Pommier Y. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity, Cancer Res 1989;49:1465-9.

Pommier Y, Kerrigan D, Kohn KW. Topological complexes between DNA and topoisomerase II and effects of polyamines, Biochemistry 1989;28:995-1002.

Fesen M, Pommier Y. Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamycin in simian virus 40 DNA, J Biol Chem, in press.

Pommier Y, Jaxel C, Kerrigan D, Kohn KW. Structure activity relationship of topoisomerase I inhibition by camptothecin derivatives: evidence for the existence of a ternary complex, Proceedings of the Second Conference on DNA Topoisomerases as Targets for Cancer Chemotherapy, in press.

Jaxel C, Capranico G, Wassermann K, Kerrigan D, Kohn KW, Pommier Y. DNA sequence at sites of topoisomerase I cleavage induced by camptothecin in SV40 DNA, Proceedings of the Second Conference on DNA Topoisomerases as Targets for Cancer Chemotherapy, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06170-05 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Histone H2A.Z Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Christopher L. Hatch	Sr. Staff Fellow	LMPH NCI
Others:	William M. Bonner	Sr. Investigator	LMPH NCI
	Concepcion Muneses	Chemist	LMPH NCI

COOPERATING UNITS *(if any)*
Medical College of Virginia (Dr. Paul Swerdlow)

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.8	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of this project are to study the function of the evolutionarily diverged but highly conserved basal H2A isoprotein, H2A.Z, in chromatin and to study the structure and function of the gene for H2A.Z.

The human H2A.Z gene has been isolated. Its sequence has been determined with the exception of a few remaining ambiguities. The gene contains four introns and has two Alu sequences in the 5′ upstream region.

CAT constructs have been assembled from various fragments of the promoter region. Maximal activity has been obtained from a 1300 bp fragment between the translation initiation site and an upstream Alu sequence.

The H2A.Z cDNA is being incorporated into the yeast S. cere. to study the function of this protein by substituting it for the yeast H2A.

PHS 6040 (Rev 1/84)

GPO 914-918

Project Description

Introduction:

The histone H2A family, unique among the four core histone families contains three species that have been maintained as separate sequences throughout evolution. The differential functions of these three core histone H2A species are unknown. In addition, the expression of different subsets of histone isoprotein genes is related to the state of cell proliferation and cycling. Although the synthesis of H2A.Z is not linked to DNA replication, it is regulated with a 20 fold difference in expression between cycling and quiescent cells. In this regard, its regulation is perhaps more similar to that of proteins such as the myc oncogene product than S-phase histones.

Objectives:

The objectives of this project are twofold. The first and ultimate objective is to study the function of the evolutionarily diverged but highly conserved basal H2A isoprotein, H2A.Z, in chromatin.

The second is to study the structure and function of the gene for H2A.Z. Investigating and learning to manipulate the expression of H2A.Z in various cells and tissues will help elucidate both
the regulation of the expression of an interesting gene and the functioning of a protein of fundamental importance in chromatin.

Methods:

1. Recombinant DNA techniques.

2. Use of two dimensional gel electrophoresis for identification of histone variant proteins.

Major Findings and Accomplishments:

1. Isolation of Human H2A.Z Gene

We have sequenced the H2A.Z cDNAs from three different mammalian species; human, cow, and rat. Those sequences have been published. The human H2A.Z gene has been isolated. Its sequence has been determined with the exception of a few remaining ambiguities. The gene contains four introns and has two Alu sequences in the 5′ upstream region.

2. Regulation of H2A.Z Gene Expression

CAT constructs have been assembled from various fragments of the promoter region. Maximal activity has been obtained from a 1300 bp fragment between the translation initiation site and an upstream Alu sequence. The mRNA level of H2A.Z decreases 20 fold when proliferating cells become quiescent. We are at present analyzing the regulation of H2A.Z expression during different states of cell growth and proliferation.

3. Yeast Studies on the Functional Role of H2A.Z

While H2A.Z seems to be the H2A isoprotein that is least similar to its counterparts, its sequence also appears to have been highly conserved during evolution. The histone H2A.Z cDNA's from human, cow, and rat encode identical proteins. The cDNA's for the H2A.Z homologous gene has been identified in chicken, sea urchin, Tetrahymena, and Drosophila. The overall similarity between mammalian and urchin H2A.Z is 95%, but there is only a 59% homology between mammalian H2A.Z and H2A.1. In order to investigate possible functions of H2A.Z, we have used yeast, Sac charomyces cerevisiae, as it is a lower eukaryote which has homologous core histone proteins but no homologue to H2A.Z, and the organism is highly genetically manipulable. In order to expedite this approach we have arranged to collaborate with Dr. Paul Swerdlow, Medical college of Virginia, Virginia Commonwealth University, Richmond, Virginia. He had already conducted an investigation into the role of H2A-ubiquitin conjugate formation on the phenotype of this organism. In order to do so he utilized a strain of Saccharomyces cerevisiae which had been made defective in the function of its two H2A genes and was dependent on the presence of H2A gene(s) maintained on extrachromosomal plasmids. He has sent us a yeast extrachromosomal plasmid which contains the normal yeast H2A promotor region and we have cloned into this plasmid the gene encoding mammalian H2A.Z (bovine nucleotide sequence) and the gene encoding human H2A.1. He is at present introducing these plasmids into the yeast H2A-mutant strain and will analyze the phenotypic characteristics of the resultant mutants. We will analyze the histone variants expressed on two-dimensional gels and we will analyze both histone messenger RNA levels as well as the levels of a variety of other mRNA's. Although it is difficult to anticipate the results, if yeast mutants containing either a major mammalian H2A variant (H2A.1) or a minor mammalian H2A variant (H2A.Z) are viable and grow normally but do show defined differences in phenotype and genetic expression then we will be able to construct hybrid major-minor variant H2A genes in order to localize the polypeptide sequences which afford the two classes of histone variant proteins different capabilities in genetic regulation.

Proposed Course:

1. Continue to study the genomic organization of the H2A.Z gene. Determine the copy number of the functional gene within the human genome and whether or not processed pseudogene copies are present.

2. Identify regulatory elements within the promoter and perform footprinting and gel retardation assays to identify regulatory proteins and their sites.

3. Search for cytoplasmic factors which specifically bind minor histone variant mRNA's to affect their stability and translation in cycling and quiescent cells.

4. Continue a collaboration with Dr. Paul Swerdlow of the Medical College of Virginia in which we will utilize yeast as a model system to study the phenotypic characteristics and genetic activity of mutants which express and utilize only either a major histone variant H2A (human H2A.1) or a minor histone variant (mammalian H2A.Z).

Publications:

Hatch CL, Bonner WM. Sequence of cDNAs for mammalian H2A.Z, an evolutionarily diverged but highly conserved basal histone H2A isoprtein species, Nucleic Acids Res 1988;16:1113-24.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06171-05 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chromatin Synthesis and the Control of Cell Proliferation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William Bonner	Sr. Investigator	LMPH NCI
Others:	Nancy Touchette	Sr. Staff Fellow	LMPH NCI
	Christopher Hatch	Sr. Staff Fellow	LMPH NCI
	Concepcion Muneses	Chemist	LMPH NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.7	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our objective is to understand the relationship of various cell states and the transitions between them as reflected in the control of histone and chromatin biosynthesis. Currently we are investigating the transition of cells from the cycling state to the quiescent state, using hamster ovary (CHO) cells that can be synchronized.

We are using this system to investigate and characterize changes involved at the protein, mRNA, and gene level during this transition. One major question concerns the relationship of the cycling-quiescent transition to the reverse transition, in terms of the expression of oncogene as well as other genes. Present studies are focusing of the mRNA for H2A.Z, a basal histone the synthesis of which is not linked to DNA replication but is 20 fold lower in quiescent relative to cycling cells. One goal is to isolate factors that regulate transcription or mRNA stability during this transition.

Project Description

Introduction:

The biochemical mechanisms which regulate cell proliferation remain largely unknown. The rate of protein synthesis in quiescent cells in approximately 1/3 that of cycling cells. When S phase CHO cells are put into media that leads to the quiescent state, the ongoing S phase continues at an undiminished rate. Currently we are investigating the transition of cells from the cycling state to the quiescent state, using hamster ovary (CHO) cells that can be synchronized through the cell cycle before entering quiescence.

Objectives:

1) The development of a greater understanding of the relationship of mechanisms regulating cell proliferation, with emphasis on the relationship of the chromosome cycle to the transition between the cycling and quiescent states.

2) A characterization of the responses of these regulatory mechanisms to the development of new regimens or compounds that might control in appropriate proliferation of transformed cells.

Methods:

(1) Discontinuous electrophoretic separation of histones. (Methods developed in this laboratory).

(2) Synchronization of cell lines, particularly Chinese hamster ovary cells for studies on cell cycle.

(3) Maintenance of cells in viable non-dividing states using modified and defined media.

(4) Isolation and analysis of mRNA levels from different parts of the cell cycle or from quiescent cells.

(5) Nuclear runoff assays to measure rates of mRNA transcription.

(6) FACS analysis of cell cycle distributions including the BrdU antibody technique for measuring S phase cells.

Major Findings and Accomplishments:

Relationship Between the Chromosome and Growth Cycles during the Transition from the Cycling to the Quiescent State

Previously, we have found that the level of histone mRNAs increases in response to moderate inhibition of protein synthesis to an extent sufficient to offset most of that inhibition, and have named this phenomenon chromosome cycle compensation. Quiescent cells have rates of protein synthesis which are about 1/3 of that found in cycling cells. We have shown

that when CHO cells, synchronized in or at the beginning of S phase, are transferred to serum-free growth media, the rate of protein synthesis starts decreasing almost immediately to the rate found in quiescent cells; however, the progression of the cells through S phase is not decreased. These results suggest that some parameters in the cell are already changing to that found in the quiescent state, even though the chromosome cycle is still in progress. Chromosome cycle progression could be maintained by the compensation mentioned above.

In order to test this hypothesis, the level and rate of transcription of certain mRNAs are being measured with respect to the rate and timing of their decrease. Present work is concentrating on the mRNA for H2A.Z, a basal histone, the synthesis of which is independent of DNA synthesis but which is decreased by 20 fold in quiescent relative to cycling cells. We have found that H2A.Z mRNA does not decrease until about 6 hr after S phase has finished.

Significance:

Cancer cells grow and divide inappropriately. Understanding the mechanism regulating the transitions between the growth and quiescent states may lead to greater understanding of this inappropriate growth. While much attention has been focused of growth inducing substances, less has been focused on quiescence inducing substances. The inability to produce a quiescence inducing substance may transform a cell just as easily as the inappropriate production of a growth inducing substance. One aim of this study is to compare the two transitions, and thus gain insight into their similarities and differences.

Proposed Course:

1. To measure the level and rate of transcription of various mRNAs, including those for H2A.Z and H2A.X during the transitions from cycling to quiescence and vice-versa.

2. To extend the above studies to include mRNAs of known oncogenes and other proteins of potential interest.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06172-05 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 charecters or less Title must fit on one line between the borders.)*
Molecular Mechanism of Action of Antitumor Alkylating Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Kurt W. Kohn	Lab Chief	LMPH NCI
Others:	Patrick O'Connor	Visiting Fellow	LMPH NCI
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	Karsten Wassermann	Visting Fellow	LMPH NCI
	Giovanni Capranico	Guest Researcher	LMPH NCI

COOPERATING UNITS *(if any)*
Drug Synthesis and Chemistry Branch, DTP, DCT, NCI (Drs. V. Narayanan, R. Haugwitz and E. Acton; Pediatric Branch, DCT, NCI (Dr. Ian Magrath)

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.7	1.7	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ability of nitrogen mustards to react selectively with particular DNA sites, depending on DNA sequence and conformation, is being studied as a possible new avenue for the design of more selective drugs. The first objective is to understand at the chemical structure level the factors that govern the selectivity for certain DNA alkylation sites. In a survey of 35 additional nitrogen mustards from the inventory of the Developmental Therapeutics Program, 2 new structures were identified that have unique reaction selectivities at the DNA sequence level. The nature and origin of these unique selectivities are being investigated, in order to obtain additional leads on how DNA sequence selectivities could be built into specifically designed new drugs. Studies to determine the DNA sequence selectivity of the reactions with DNA in nucleosomal chromatin and in cell nuclei are in progress. The mechanism and role of DNA interstrand cross-linking in the action of nitrogen mustards is being reinvestigated in view of new data that suggest that some previously held hypotheses may be inaccurate. The kinetics of formation and repair of DNA interstrand and DNA-protein cross-links in mammalian cells have been determined for several nitrogen mustards, and have disclosed substantial differences that may be of pharmacological importance in regard to the relative clinical effectiveness of nitrogen mustard derivatives.

PHS 6040 (Rev 1/84) GPO 914-918

Proect Description

Objectives:

1. Determine the structural basis for the nucleotide sequence selectivity of DNA alkylation reactions.

2. Design new alkylating agents having optimized DNA sequence selectivities.

3. Investigate the possibility of using this approach to enhance the specificity of antitumor action.

4. Determine the kinetics of DNA interstrand and DNA-protein crosslink formation and repair in cells in relation to cytotoxic consequences.

5. Investigate possible relationships between DNA sequence-selectivity of alkylation reactions, the formation of DNA crosslinks and the sensitivity of cells to nitrogen mustards.

Methods:

1. Application of DNA sequencing gel electrophoretic techniques to the quantitation of extents of alkylation at specific sites in a sequence.

2. Standard techniques of cellular and molecular biology.

3. DNA filter elution techniques to quantitate DNA lesions in cells.

DNA sequence selective alkylating agents

We have been investigating the DNA sequence selectivity of the sites of DNA alkylation by nitrogen mustards, in order to investigate the possibility that the preferred sites of alkylation would influence the anticancer effectiveness of these drugs. Alkylation at guanine-N7 sites was studied because these are the predominant sites of alkylation by nitrogen mustards in DNA and are the sites likely to be involved in the formation of potentially lethal crosslinks. Also, the alkylated guanine-N7 positions are readily converted to DNA strand breaks which can be measured by DNA sequencing methodology. Using uniquely end-labeled DNA fragments, we had found that differences in alkylation intensities do exist among different sites and that the range of variation in dilute solution is at least a factor 10 to 20. The DNA sequence dependence of the alkylation intensities of most nitrogen mustard was similar and the analysis suggested that the selectivity was mainly due to differences in molecular electrostatic potential in the vicinity of the guanine-N7 alkylation site, dependent upon influences from the neighboring base pairs. Some nitrogen mustards, including uracil mustard and quinacrine mustard, however, exhibited exceptional behavior and demonstrated that the preferred sites of alkylation can be influenced by alterations in the structure of the nitrogen mustard. This was an encouraging result with respect to the possibility that new nitrogen mustards could be designed having optimum selectivity for reaction with those sequence sites that would be most effective in producing DNA lesions that contribute to the anticancer activity.

The previous work however was carried out in dilute aqueous solution, leaving open the question of whether or not the reaction preferences would be markedly altered in the cell chromatin. We have examined the effects of salt composition and polyamines, and found that these ions reduce the magnitude of that part of the sequence selectivity that was attributable to electrostatic potential. The specific sequence preferences exhibited by uracil mustard however were less affected and, in the case of quinacrine mustard, were essentially unaffected. We are investigating the effect of nucleosome reconstitution. Early results suggest that there is surprisingly little effect on the selectivity pattern.

Of the 7 commonly used or extensively studied nitrogen mustards that we had selected for the original DNA sequence selectivity investigation, 2 showed unique differences which, in both cases, could be rationalized on the basis of molecular structure. In view of this seemingly high yield of enlightening compounds, we screened an additional 35 nitrogen mustards selected from the DTP inventory. Two compounds exhibiting new unique patterns of alkylation were found. One is a guanine mustard and the other is an unfused tricylic compound. We are investigating these compounds in order to obtain new clues as to how sequence selectivity could be built into nitrogen mustard structures.

DNA lesions in cells treated with nitrogen mustards

It is generally agreed that DNA interstrand crosslinks are the most likely cytotoxic lesions produced by alkylating agents. DNA structural considerations suggested that the site that would most easily lead to an interstrand crosslink would be in the sequence 5-GC-3′. For most nitrogen mustards, these are weak alkylation sites; for uracil mustard, however, some of these sites are very strong. Therefore we tested the possibility that uracil mustard might be unusually efficient in producing interstrand crosslinks. We found, contrary to expectation, that this is not the case. There are several possible reasons for this, including the possibility that 5′-GC-3′ may in fact not be the main crosslinking site. We are now pursuing this question.

In order to obtain reliable comparisons of interstrand crosslinking efficiency, kinetic experiments were carried out on the formation and repair of both interstrand and DNA-protein crosslinks using several nitrogen mustards. Sufficient quantitative data was obtained, using DNA alkaline elution methodology, to permit estimation of the kinetic constants for crosslink formation and repair. We expected that the ratio of interstrand relative to DNA-protein crosslinking would be increased in the case of uracil mustard. A close chemical relative, 6-methyl uracil mustard, was available for comparison, since this derivative lacks the special sequence preferences exhibited by the parent compound. The kinetics for the two compounds were found to be similar. The ratio of interstrand to DNA protein crosslinking, however, was increased, rather than decreased, for the 6-methyl derivative.

These experiments also showed that there are substantial differences among nitrogen mustards in the kinetics of crosslink formation and repair. The area under the interstrand crosslinking-versus-time curve was found to

correlate with cell killing in colony survival assays. These molecular pharmacokinetic differences are additional parameters upon which the effectiveness of new nitrogen mustards could be optimized. It may be possible to extend classical pharmacokinetic analysis to include the formation and removal of DNA crosslinks, which could be treated as additional compartments in a comprehensive model.

We had entertained the simple hypothesis that the antitumor effectiveness of nitrogen mustards may in part be due to preferential crosslinking of DNA regions that are rich in guanines. This possibility seemed favored by the fact that the eukaryotic genome contains large regions that are extremely GC-rich, and by our finding that nitrogen mustards have a strong preference for reaction within clusters of G's, which would have a high expectancy of occurrence in GC-rich regions and where there would be the potentiality for the formation of intra-strand crosslinks. We have explored this possibility in Burkitt's lymphoma cell lines containing the Ebstein-Barr virus (EBV) genome, much of which is extremely GC-rich, since this lymphoma has shown extraordinary sensitivity to alkylating agents. We have studied 8 Burkitt's lymphoma cell lines obtained from the Pediatric Oncology Branch, containing known chromosome translocations. Four of the lines harbored the EBV genome and 4 did not. We tested the hypothesis that the presence of the highly GC-rich EBV genome would enhance sensitivity to nitrogen mustards. The results showed that, although there were substantial differences in drug sensitivity among the cell lines, there was no correlation with the presence or absence of the EBV genome, nor was there any correlation with the type of chromosome translocation present in the cell lines. The origin of the sensitivity differences between genomically matched pairs of cell lines can now be investigated.

Significance

Our results have continued to be encouraging with respect to the possibility that new DNA sequence-selective alkylating agents could be designed for improved antitumor selectivity. New leads have been obtained for structures that confer DNA sequence selectivity of alkylation. Some of our initial simple hypotheses regarding interstrand crosslinks however have not been substantiated by our current experiments. The formation and significance of DNA crosslinks will be reinvestigated on the basis of alternative hypotheses.

Proposed Course:

1. Follow up on new leads to determine the basis for the DNA sequence selectivities of the new specific nitrogen mustards that we have detected. Define the nature of the new selectivities and attempt to account for them on a structural basis.

2. Investigate new intercalating mustards that are now being synthesized. This is a follow-up of results obtained with quinacrine mustard and older reports that such compounds are unusual in that the monofunctional nitrogen mustard derivatives have good antitumor activity.

3. Determine the effect of nucleosomes on the DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards.

4. Establish methodology for the determination of alkylation sites in cell nuclei.

5. Study DNA interstrand crosslink formation in synthetic oligonucleoides of defined sequence.

Publications:

1. Hartley JA, Lown JW, Mattes WB, Kohn KW. DNA sequence specificity of antitumor agents. Oncogenes as possible targets for cancer therapy, Acta Oncol 1988;27:503-10.

2. Mattes WB, Hartley JA, Kohn KW, Matheson DW. GC-rich regions in genomes as targets for DNA alkylation, Carcinogenesis 1988;9:2065-72.

3. Kohn KW, Hartley JA, Mattes WB. Mechanisms of DNA sequence selective alkylation of guanine-N7 positions by nitrogen mustards, Biochem Pharmacol 1988;37:1799-800.

4. Hartley JA, Mattes WB, Vaughan K, Gibson NW. DNA sequence specificity of guanine N7-alkylations for a series of structurally related triazenes, Carcinogenesis 1988;9:669-74.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06186-03 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Repair at the Level of the Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vilhelm Bohr	Medical Officer	LMPH	NCI
Others:	Diane S. Okumoto	Microbiologist	LMPH	NCI
	Karsten Wassermann	Visiting Fellow	LMPH	NCI
	Michele Evans	Medical Staff Fellow	LMPH	NCI
	Jennifer Jones	General Fellowship	LMPH	NCI
	Tina Stevnsner	Guest Researcher	LMPH	NCI
	Wei-Ping Zhen	Visiting Fellow	LMPH	NCI
	Jeffrey Beechman	Graduate Student	LG	NCI

COOPERATING UNITS *(if any)* University of North Carolina (David C. Thomas); M. D. Anderson Tumor Hospital (Moon-Song Tang); NIH (Jay Robbins, Michael Gottesman, Snorri Thorgeirsson); Georgetown University (Mark Smulson)

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our objective is to study the DNA damage and repair in various individual genes and in non-coding sequences with in the genome. This is correlated to more traditional studies of DNA repair as an average over the genome. Findings reviewed in Bohr VA et al. Cancer Res 1987;47:6426-6436, have indicated that active genes are preferentially repaired in mammalian cells and that determinations of DNA repair in specific genes are important for correlations to biological endpoints and risk assessments. Whereas our earlier studies were limited to UV as a damaging agent, we have now developed approaches for studying damage and repair after a number of carcinogens that react with the DNA.

We are studying the DNA repair of genes in a number of human, cancer prone DNA repair deficient syndromes and in various human and rodent mutant cell lines, some of which are transfected with repair in genes.

GPO 914-918

Objectives:

1) To study DNA damage and repair in specific genes using established methodology. To develop new methodology to examine DNA damage and repair after instructural genes and oncogenes following treatment with various carcinogens.

2) Examine DNA repair of various genes in DNA damage sensitive human cell lines and in various rodent cell lines.

3) Develop assays to study DNA damage and repair in specific genomic sequences in human primary cell cultures and patient material.

4) Further explore the molecular rules that govern preferential repair of genes in normal and repair deficient mammalian cells.

Methodology:

1) Cell culture techniques.

2) DNA damage and repair.

3) Drug pharmacology.

4) DNA molecular techniques, molecular hybridizations.

5) RNA techniques.

6) Hybridization and quantitation.

7) General molecular biology.

8) Centrifugation techniques.

9) Densitometry.

10) Plasmid analysis.

11) Computer programming.

Brief Summary of Previous Results:

We have demonstrated that essential genes in rodent and human cells are preferentially repaired after UV damage. In rodent cells, some genes are repaired much more efficiently than the bulk of the genome. This may explain the long standing paradox that the overall genome repair capacity of rodent cells is low, whereas their UV survival is as high as for proficiently repairing human cells: Rodent cells appear to repair efficiently only genomic regions of vital importance. In normal human cells, we have found that genes are repaired faster than the bulk of the genome. We have demonstrated that determinations of DNA repair in specific genomic sequences may be more important than overall genome DNA repair measurements for correlations to other biological end points such as resistance to UV damage. Changes in preferential DNA

repair could have profound effects on such parameters without noticeably altering overall genome repair levels since the vital regions only constitute a very small fraction of the genome. We have analyzed the genomic fine structure of DNA repair in and around the DHFR gene in CHO cells and find a region of preferential DNA repair (DNA repair domain) of approximately 60-80 kb in length with maximal DNA repair efficiency at the 5′ end of the gene and in its 5′ flanking sequences. This size corresponds very well with proposed and measured lengths for loops or domains of higher order structure in chromatin, and suggests that DNA repair efficiency in specific genomic regions might reflect aspects of local chromatin structure and thus provide us with a probe for the detection of chromatin structural changes.

We have found considerable differences in the repair efficiency of different genes within the same cell. The constitutively transcribed protooncogene c-abl is much more efficiently repaired than the transcriptionally silent proto-oncogene c-mos. In experiments measuring the repair in genes which can be modulated with regard to activity, results have further suggested that when metallothionein genes which are normally inactive become activated, considerable corresponding increases in DNA repair efficiencies can be detected. These findings suggest a positive correlation between DNA repair efficiency and transcriptional activity in a gene.

We have studied the effect of the topoisomerase II inhibitor novobiocin on repair in the overall genome and in the DHFR gene. Whereas this compound inhibits overall genome repair, it had no effect on repair in the gene. This suggests that qualitative as well as quantitative differences exist between the "average" repair pathway in the cell and that responsible for the preferential repair seen in active genes.

MAJOR FINDINGS:

1) Repair of genes in human disease.

A number of human disorders have been termed DNA damage sensitive or repair deficient syndromes. For none of these is the etiology of the disease known, nor is it known which aspect of the DNA repair mechanism is deficient. We have analyzed DNA repair in specific genes in particularly cancer prone DNA damage sensitive or repair deficient syndromes: Dysplastic nevus syndrome, Bloom syndrome, Cockaynes syndrome and Gardner's syndrome. Preferential DNA repair after UV damage was found in Dysplastic Nevus syndrome and Bloom syndrome, but it was deficient in Cockayne's syndrome and in Gardner's syndrome. The lack of preferential DNA repair in the latter two diseases is of considerable interest, since it is the first demonstration of a human cell line not capable of performing preferential DNA repair.

We have recently studied the repair of the essential DHFR gene in some xeroderma pigmentosum (XP) complementation groups and found that we could differentiate between 3 levels of DNA repair efficiency in the gene. Gene repair was determined in normal human cells, XP group C (2 lines), XP group A and XP group F. The relative levels of overall genome repair in these cells were: normal > XPC > XPF > XPA. The relative levels of UV resistance were: normal > XPF > XPC > XPA. In the analysis of repair of UV dimers in the gene, we found

the relative efficiencies: normal > XPC > XPA > XPF. The XPF cell line is of particular interest since it has high survival, but low overall genome removal of pyrimidine dimers. We had expected a high level of gene repair in XPF, but found very little. This suggests that the survival could be linked to the efficient repair of an adduct other than pyrimidine dimers. We are currently examine the repair of other adducts in the DHFR gene in these human repair deficient cells.

2) Effect on gene repair of inhibitors of topoisomerases and polymerases.

It is possible that the preferential DNA repair in individual genes is carried out by specific enzymes different from those responsible for the average overall genome DNA repair. We have therefore tested the effect of specific enzyme inhibitors on the repair in the CHO DHFR gene as well as on the overall genome in CHO cells after UV damage. The alpha DNA polymerase inhibitor aphidicolin which inhibits overall genome repair in some cell lines, but not in others had no effect on overall repair in the CHO cells. Also, It had no effect on repair in the DHFR gene. The topoisomerase II inhibitor merbarone did inhibit the overall genome repair by about 30%, but did not affect the repair in the DHFR gene. The topoisomerase I inhibitor camptothecin slightly inhibited the overall genome repair, but had no effect on the repair in the gene. The poly ADPr polymerase inhibitor 3-aminobenzamide had no effect on the overall genome repair and the gene repair. These inhibitor experiments did not reveal a preferential involvement of any enzyme in the repair of active gene, but we are considering some more sophisticated experiments to elucidate this issue.

3) Formation and repair of minor photoproducts in the CHO DHFR gene.

UV irradiation introduces photoproducts in the DNA. The major photoproduct (60-80%) is the pyrimidine dimer. However, other photoproducts are also formed, notably the 6-4 photoproduct (6-4 PP). Almost all the work done on UV damage and repair is based on analysis of pyrimidine dimers, and although recent data suggest that the 6-4 PP plays a major role in mutagenesis, little is known about the fate of this photoproduct. We have developed an assay to determine the formation and repair of 6-4 PP in individual genes. Using this approach, we have found that the frequency of 6-4 PP is about 40% of that of pyrimidine dimers in the DHFR gene; this frequency is higher than previously thought. We have further demonstrated that the 6-4 PP is preferentially repaired in the gene as compared to non-transcribed genomic regions.

4) DNA damage and repair in genes by drugs and carcinogens.

The major effort in my research group over the last years has been to develop new techniques to study the damage and repair of various carcinogens in specific genes. In general this approach involves gene targeted therapy, drug resistance and the molecular biology of DNA repair in genes. With the use of the ABC excinuclease, we have developed methodology to quantitate cisplatinum, 4NQO, IQ (a highly mutagenic, heterocyclic compound) and NAAAF. We have been able to demonstrate preferential DNA repair of cisplatinum adducts in the CHO DHFR, whereas NAAAF adducts were not. In addition an assay has been developed to directly detect the sites of alkylation damage in DNA. In this assay, the the DNA is nicked at alkylated sites by depurination and endonucleolytic

cleavage, and the frequency of alkylated sites in genes can then be probed. Using this approach, we have recently found that adducts formed after treatment with nitrogen mustard are preferentially repaired in the CHO DHFR gene.

PROPOSED COURSE:

The objective is to further investigate aspects of preferential DNA repair in genes including its implication in cancer therapy and diagnosis.

There are 2 overall directions in our research program: 1) the further investigations of the molecular biology of preferential DNA repair, and 2) DNA repair in human disease, relation to cancer risk and therapy.

Molecular biology of preferential DNA repair in genes.

There are a number of issues we wish to investigate in the long term to reach a better understanding of the properties of repair in genes. They include the importance of the transcriptional state or level of a given gene, the "type" of gene (essential, structural, regulated, stage of differentiation), a number of chromatin structural elements (accessibility, ADP ribosylation, methylation) and the type of topoisomerase and DNA polymerase involved. We are currently investigating some of these relationships: We are looking at a variety of genes for their repair, some of them can be modulated with regard to transcriptional activity and some of them are at different developmental or differentiational stages. We have recently studied the importance of the level of methylation in individual genes and at the level of the overall genome, and we are currently pursuing this. We are also interested in the question of whether repair in genes is dealt with by specific enzymes as a contrast to the repair in the overall genome. We are examining the repair of various types of damage in CHO mutant cell lines containing some of the recently cloned human DNA repair genes.

Human disorders and cancer risk.

Preferential DNA repair is presently being examined in a newly established xeroderma pigmentosum cell line which is transfected with the bacterial repair gene, denV. This cell line represents the first transfectant of a xeroderma cell line with a repair gene, and thus an example of gene therapy. We are examining the repair in a number of genes in various human disorders, some that are suspect for repair deficiency, some that are associated with immunological deficiencies (e.g. Bloom syndrome), and some that are associated with neurological deficiencies (e.g. Alzheimer's). We are particularly interested in the potential relation between the repair in specific genes and the risk of cancer. To this end we are studying the repair at the gene level and at the overall genome level in diseases associated with very high cancer risk. One such disorder is Gardner's syndrome. Since our preliminary results have shown that this disease shows less than normal preferential repair, we are currently examining the repair of this diseases at different levels in much more detail and in at least two cell lines to further investigate the interesting indication that lack of preferential DNA repair is associated with increased cancer risk.

We are planning to study the repair in a number of interesting genes including the MDR 1 gene, the topoisomerase II gene, the poly ADP ribose gene and the beta-DNA polymerase gene.

Cancer therapy.

Anti-cancer drugs are in most instances known to interact directly with the DNA, and the frequency of such sites can thus be directly measured. This would allow us to screen a number of compounds in order to find those that bind most strongly to active genomic regions including specific proto-oncogenes. Drugs with high affinity for genes may be candidates for anti cancer therapy since inactivation of certain genes are likely to be main targets of the therapy. And since the active parts of the genome only constitute a very minor fraction of the genome (< 1%), drug affinity to those regions might increase the therapeutical efficiency dramatically.

We are further planning to examine the damage and repair in various genes of a number nitrogen mustards, of X-rays and other forms of ionizing irradiation, of oxidative damage, of heterocyclic compounds and of bleomycin and neocarcizinostatin.

Relevance to Institute goals.

Much research evidence supports that DNA repair plays a major role in the prevention of cancer and in drug resistance. Our continuous work on the fine structure analysis of DNA should further our understanding of molecular events involved in the carcinogenic process. If we can find inhibitors of the repair process, it may be very important in clinical therapy of drug resistant tumors. Our work on the repair in human syndromes may elucidate important molecular events that are part of the mechanism of the disease. In our studies of the DNA damage and repair of anticancer therapeutics, we hope to find drugs that are gene targeted and thus more selective and effective in therapy.

Publications:

Bohr VA, Phillips DH Hanawalt PC. Heterogeneity of DNA damage and repair in the mammalian genome, Cancer Res 1987;47:6426-36.

Bohr VA. Differential DNA repair within the genome, Cancer Rev 1987;7:28-55.
Bohr VA. Introduction (to issue on DNA repair), Cancer Rev 1987;7:1-3.

Okumoto DS, Bohr VA. DNA repair in the metallothionein gene increases with transcriptional activation, Nucleic Acids Res 1987;15:10021-31.

Bohr V A, Okumoto DS. Analysis of frequency of pyrimidine dimers in specific genomic sequences. In: Hanawalt PC, Friedberg, EC, eds. DNA repair: A Laboratory Manual of Research Procedures. New York: Marcel Dekker, 1988;347-66.

Thomas DC, Morton A G, Bohr VA, Sancar A. A general method for quantitating bulky adducts in mammalian genes, Proc Natl Acad Sci USA 1988;85:3723-7.

Bohr VA, Chu EHY, van Duin M, Hanawalt PC, Okumoto DS. Human repair gene restores normal pattern of preferential DNA repair in repair defective CHO cells, Nucleic Acids Res 1988;16:7397-403.

Bohr VA. DNA repair and transcriptional activity in genes, Commentary for J Cell Sci 1988;90:175-8.

Bohr VA, Wassermann K. DNA repair at the level of the gene, Trends Biochem Sci 1988;13:429-33.

Bohr VA, Hanawalt PC. DNA repair in genes, Pharmacol. Ther 1988;38:315-9.

Ho L, Bohr VA, Hanawalt PC. Demethylation enhances removal of UV damage from the overall genome and from specific DNA sequences in CHO cells, Molec Cell Biol 1989;9:1594-1603.

Bohr VA, Evans MK, Fornace AJ Jr. DNA repair and its pathogenetic implications, Lab Invest, in press.

Tang MS, Bohr VA, Zhang Xs, Pierce J, Hanawalt PC. Quantitation of aminofluorene adduct formation and repair in defined DNA sequences in mammalian cells using UVRABC nuclease, J Biol Chem, in press.

Bohr VA, Evans MK. DNA repair in individual genes, Implications for oncology, J Pediatr Oncol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06192-01 LMPH

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Histone H2A.X

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William Bonner	Sr. Investigator	LMPH NCI
Others:	Cecilia Mannironi	Visiting Fellow	LMPH NCI
	Christopher Hatch	Sr. Staff Fellow	LMPH NCI
	Concepcion Muneses	Chemist	LMPH NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The primary objective of this project is to isolate the cDNA and gene for human histone H2A.X. A secondary objective is to isolate cDNAs for the H2A.1 isoprotein species that are not subject to S-phase regulation.

We have isolated the cDNA for human H2A.X. This H2A related protein which has not been sequenced previously has yielded some intriguing information concerning the relationship of the H2A histone during evolution. Seven of the final carboxy-terminal amino-acids are identical to the H2A from S. cere. There is homology with the carboxy-terminal sequences of at least one H2A in all studied lower eukaryotes.

In addition, we have isolated some cDNAs for H2A.1 subspecies. The RNAs of these subspecies are believed to be cell cycle independent.

The significance of these recent results lies first in the evidence concerning the relationship of chromatin from eukaryotes, second in the possibility of performing studies on the function of H2A.X, and third, in the mechanism of regulation of this RNA in quiescent as well as proliferating cells.

Project Description

Introduction:

Mammalian cells contain four histone H2A isoprotein species, called H2A.1, H2A.2, H2A.Z and H2A.X. In proliferating cells, the former two are synthesized in concert with chromatin replication, while the latter two are synthesized throughout the cell cycle. In quiescent cells, all four isoprotein species are synthesized at much lower rates but in relative amounts similar to those in proliferating cells. H2A.X represents about 10-15% of total H2A in most mammalian cell lines and tissues, but has been reported to be a somewhat larger fraction in testes and brain. Evidence from analysis of tryptic peptides suggests that the H2A.X and H2A.1 sequences are similar in the amino-terminal and central regions, but that they differ considerably in the carboxy-terminal region.

Objectives:

The primary objective is the isolation and characterization of the human histone H2A.X cDNA and gene. We have cloned and sequenced the cDNA complements of the gene for the histone isoprotein H2A.X from human. Although the synthesis of H2A.X, like the synthesis of H2A.Z, is not linked to DNA replication, it is still regulated with a 20 fold difference in expression between cycling and quiescent cells. In this regard, its regulation is perhaps more similar to that of proteins such as the myc oncogene product. The gene for the major histone H2A variant, H2A.1, has been isolated from a human genomic-lambda library and this gene has been utilized to isolate a number of cDNA clones potentially encoding other mRNAs for H2A.1 or H2A.2 isoproteins. These clones will soon be analyzed by subcloning and sequencing.

Methods:

1. Recombinant DNA techniques.

2. Use of two dimensional gel electrophoresis for identification of histone variant proteins.

Major Findings and Accomplishments:

1. Isolation of the cDNA for Histone H2A.X, Deduced Protein Sequence

A cDNA clone that directs the in vitro synthesis of human histone H2A isoprotein H2A.X has been isolated and sequenced. H2A.X is found to contain 142 amino acid residues, 13 more than human H2A.1. The sequence of the first 120 residues of H2A.X is almost identical to that of human H2A.1. The sequence of the carboxy-terminal 22 residues of H2A.X is unrelated to any known sequence in vertebrate histone H2A; however, it contains sequences homologous with H2A carboxy-terminal sequences of several lower organisms. This homology centers on the carboxy-terminal tetrapeptide which in H2A.X is SerGlnGluTyr. Homologous sequences are found in H2As of three types of yeasts, in Tetrahymena and Drosophila. Seven of the nine carboxy-terminal

amino acids of H2A.X are identical with those of S. cerevisea H2A.1. It is suggested that this H2A carboxy-terminal motif may be present in all eucaryotes.

2. Analysis of Factors Which Affect Histone mRNA Utilization and Metabolism

A major component in the stability of mRNA's for the replication-linked histone isoproteins is inherent in the message structure. In general, mRNA's for replication-linked histone isoproteins contain only short 5' and 3' untranslated regions, are not polyadenylated, and contain a conserved stem-loop structure at the 3' end of the mRNA. In contrast, the mRNA's of the basal histone isoproteins appear to have longer 3' untranslated regions with a terminal poly A tail and do not contain the stem-loop structure at the 3' end of the mRNA. The H2A.X cDNA is 1582 bases long followed by a polyA tail. There are 72 nucleotides in the 5' UTR, 529 in the coding region, and 1078 in the 3' UTR. Even though H2A.X is a basal or replication-independent histone, the 3' UTR of its mRNA contains a sequence with dyad symmetry closely related to the highly conserved sequences shown to be involved in the linkage between the stability of replication-dependent histone mRNAs and the rate of DNA synthesis. There is another 3' UTR sequence with dyad symmetry much further downstream. Various characteristics of the cDNA suggest that the gene is present in a CpG island in chromatin.

3. The Search for other mRNAs Encoding the Major Histone Variant, H2A.1

We have isolated cDNA colonies positive for hybridization to the human H2A.1 gene is that we may identify a class of major H2A variant genes which produce polyadenylated mRNA's and are subject to a different mode of regulation than their counterparts which are DNA replication linked in their synthesis and stability. Approximately 20 cDNA clones positive for hybridization to the human H2A.1 gene probe have been isolated and are being analyzed. Although the sequences are not yet complete, we have enough information to show that none of these cDNAs are the cDNA for the S-phase regulated H2A.1.

Significance to Biomedical Research and to the Program of the Institute

H2A.X is a member of the H2A histone family that has retained a conserved carboxy-terminal motif throughout evolution. This research will help elucidate the control of chromatin function not only during replication in proliferating cells but also in quiescent cells.

Proposed Course:

1. Characterize the expression of the cDNA for H2A.X. Compare its expression to that already known for H2A.1 and H2A.Z. These studies should be particularly interesting because the H2A.X cDNA contains the regulatory sequence for S-phase regulation, but also contains polyA and is not S-phase regulated.

2. Isolate the genomic form of H2A.X.

3. Sequence the isolated H2A.1 cDNA clones, with particular attention to the processing signals in the 3' region of the cDNAs.

Publications:

Mannironi C, Bonner WM, Hatch CL. Human H2A histone isoprotein H2A.X has carboxy-terminal sequence homologies with H2As of lower eukaryotes; cDNA and derived protein sequences, Nucleic Acids Res, in press.

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