tober 1, 1989–September 31, 1990

Annual Report

Division Of

Cancer Treatment

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NATIONAL CANCER INSTITUTE (U.S.)

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ANNUAL REPORT

October 1, 1989 through September 30, 1990

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ANNUAL REPORT

DIVISION OF CANCER TREATMENT

October 1, 1989 through September 30, 1990

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 involves the investigation of chemotherapy, surgery, radiation therapy, immunotherapy and biologic response modifiers, used individually and in combination. The conduct of this research follows a logical 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 renowned scientists from 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. Important advances were made in all phases of cancer research in 1990, from the very earliest stages of drug discovery and development, where we have achieved full implementation of the new human tumor cell line screen, to the most advanced stages of drug development prior to new-drug approval, where we have focused our efforts on increasing the supply of taxol, a unique mitotic spindle poison that has demonstrated promising activity in refractory ovarian and breast cancers.

Laboratory developments in the field of AIDS research have provided important new insights into the pathogenesis and life cycle of the human immunodeficiency virus (HIV), insights that are essential for the development of effective treatment for this disease. One of the biggest challenges has been the development of therapies effective against HIV-infected monocytes, which are not actively replicating but contain latent HIV. Investigators in the BRMP have recently identified several unique DNA binding proteins that bind to the long terminal repeat (LTR) sequence

TABLE I. DCT BOARD OF SCIENTIFIC COUNSELORS

Name	Affiliation	Term of Appointment
John E. Neiderhuber, M.D. (Chairman)	Johns Hopkins University School of Medicine	1986-1991
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
Phillip Crews, Ph.D.	Univ. of California, Santa Cruz	1989-1993
Emil Frei, III, M.D.	Dana-Farber Cancer Institute	1986-1990
Mark T. Groudine, M.D., Ph.D.	Fred Hutchinson Cancer Res. Ctr.	1986-1991
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	1990-1992
Frank M. Huennekens, Ph.D.	Res. Institute of Scripps Clinic	1988-1991
Ronald Levy, M.D.	Stanford University Medical Ctr.	1989-1993
John Mendelsohn, M.D.	Memorial Sloan-Kettering Cancer Center	1986-1990
JoAnne Stubbe, Ph.D.	Massachusetts Inst. of Technology	1989-1993
Ralph R. Weichselbaum, M.D.	Univ. of Chicago Medical Center	1989-1993

of HIV and may regulate viral replication. Characterization, sequencing and molecular cloning of these molecules could open the way for more effective treatment of AIDS and other HIV-associated diseases. Clinical studies conducted in COP suggest that prolonged antiretroviral activity and improved tolerance to therapy can be obtained through combined dideoxynucleoside therapy. Phase I development of new antiretroviral therapies, such as pentosan, continues.

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, Deputy Director of the Division, has also been appointed Acting Associate Director for the Developmental Therapeutics Program.

- Ms. Kathy Russell left her position as Deputy Administrative Officer, DCT, to join the staff of Georgetown University.

- Ms. Francine Little, formerly Budget Officer in the NCI's Financial Management Branch, was selected as the new Deputy Administrative Officer, DCT.

B. Biological Response Modifiers Program (BRMP)

- Dr. Ira Green left his position as Medical Officer, Biological Resources Branch, to accept an appointment with the Agency for Health Care Policy and Research.

- Dr. Robert Fenton, formerly with the Dana-Farber Cancer Institute, was recruited as an Expert in the Clinical Research Branch.

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

- Dr. William Sharfman, formerly with the Cleveland Clinic, was recruited as an Expert in the Clinical Research Branch.

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

- Dr. John O'Shea was recruited as an Expert in the Laboratory of Experimental Immunology, transferring from the National Institute on Child Health and Human Development.

- Dr. Louis Matis was recruited as a Medical Officer in the Laboratory of Molecular Immunoregulation, transferring from the Molecular Laboratory of the Center for Biologics Evaluation and Research, FDA.

- Dr. Jon T. Holmlund, formerly with George Washington Univ. Medical Center, was recruited as a Medical Officer in the Biological Resources Branch.

- Dr. Brendan D. Curti, formerly with Georgetown University Hospital, was recruited as a Senior Staff Fellow in the Clinical Research Branch.

C. Cancer Therapy Evaluation Program (CTEP)

- Dr. David R. Parkinson, formerly Chief, Section of Biological Therapy of Solid Tumors, Division of Medicine, University of Texas M.D. Anderson Hospital, was recruited as Head, Biologics Evaluation Section, Investigational Drug Branch.

- Dr. John Brennan was recruited as a Medical Officer in the Clinical Investigations Branch after serving as a Clinical Instructor at the Uniformed Services University of the Health Sciences.

- Dr. Hoo Chun, a Cancer Expert in the Developmental Chemotherapy Section, Investigational Drug Branch, left to continue his career in academic medicine.

- Paul Hiranaka, Senior Clinical/Research Pharmacist in the Drug Regulatory Affairs Section, Regulatory Affairs Branch, has retired.

- Joseph High, a Pharmacist in the Clinical Center, NIH, has transferred to the Drug Management and Authorization Section, Investigational Drug Branch.

- Dr. Langdon Miller joined the Biologics Evaluation Section, Investigational Drug Branch, as a Senior Investigator; he was formerly an Associate Investigator at the Palo Alto VA Medical Center in California.

- Dr. Henry Stevenson transferred from the BRMP to the Biologics Evaluation Section, Investigational Drug Branch.

- Dr. Malcolm Smith of the COP will be spending a year of his fellowship as a Senior Investigator in the Pediatric Section, Clinical Investigations Branch.

- Dr. Estelle Russek-Cohen, from the University of Maryland, completed a year in the Biometric Research Branch under the Interagency Personnel Act.

- Dr. Alison Martin resigned as a Senior Investigator in the Biologics Evaluation Section, Investigational Drug Branch, to join the Washington Clinic.

D. Clinical Oncology Program (COP)

- Dr. H. Richard Alexander, Dr. Richard B. Alexander, Dr. Jeffrey S. Weber, Dr. Stephen E. Ettinghausen, and Dr. Douglas J. Schwartzentruber have been appointed Senior Investigators in the Surgery Branch.

- Sir A. Patrick Forrest joined the staff of the Medicine Branch as a Visiting Scientist for one year; he comes to DCT from the University of Edinburgh, Scotland, where he is Professor Emeritus and Honorary Fellow.

- Dr. Bruce Johnson has been appointed Head of the newly established Molecular Biology of Oncopeptides Section of the NCI-Navy Medical Oncology Branch.

- Dr. Dwight Kaufman transferred from the Medicine Branch to the Radiation Oncology Branch as a Senior Investigator.

- Dr. Michael T. Lotze, Senior Investigator in the Surgery Branch, left to accept a position as Professor of Surgery, Director of Surgical Oncology and Associate Director of the Pittsburgh Cancer Institute, Univ. of Pittsburgh.

- Upon reactivation of the Clinical Pharmacology Branch, Dr. Charles E. Myers stepped down from his position as Chief, Medicine Branch, to assume the position of Chief, Clinical Pharmacology Branch.

- Dr. Robert Wittes has been appointed Chief, Medicine Branch; he returns to DCT from the Bristol-Myers Company, where he was Senior Vice President for Cancer Research.

- Dr. Lori Pierce has been appointed a Radiation Oncologist in the Radiation Oncology Branch.

- Dr. Catherine Salem has been appointed Head of the Navy Section. Radiation Oncology Branch.

- Dr. Edward Sausville joined the staff of the Medicine Branch as a Senior Research Investigator; he returns to DCT from Georgetown University, where he was Associate Professor of Medicine at the School of Medicine and attending physician at the Lombardi Cancer Center.

- Dr. Karen Straus, a Radiation Oncologist in the Radiation Therapy Section, Radiation Oncology Branch, left to go into private practice in Winchester, Virginia.

- Dr. Zelig Tochner, a Radiation Oncologist in the Radiation Therapy Section, Radiation Oncology Branch, left to return to Israel.

- Dr. Alison Freifeld joined the Pediatric Branch as a Medical Officer; she was formerly with the National Institute of Allergy and Infectious Diseases.

- Lori Weiner became a member of the Pediatric Branch senior staff as the AIDS Patient Counselor.

- Dr. Renato LaRocca, a Senior Investigator in the Medicine Branch, left NCI to enter private practice in Louisville, Kentucky.

E. Radiation Research Program (RRP)

- Dr. Faina Shtern was hired as Acting Chief of the Diagnostic Imaging Research Branch in January; she was formerly an Assistant in Radiology at the Mass. General Hospital and an Instructor in Radiology at Harvard Medical School.

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

F. Developmental Therapeutics Program (DTP)

- Dr. Michael R. Boyd, who was Associate Director for the DTP, accepted a position as Chief, Laboratory of Drug Discovery Research and Development.

- Dr. Michael R. Grever was appointed Acting Associate Director for the DTP.

- Dr. Charles Grieshaber, Chief, Toxicology Branch, transferred to the Food and Drug Administration.

- Dr. Edward Acton was appointed Deputy Chief, Drug Synthesis and Chemistry Branch.

- Dr. Robert Glazer, Pharmacologist, Laboratory of Biological Chemistry, accepted a position at Georgetown University.

- Dr. Gordon Cragg was appointed Chief, Natural Products Branch.

- Dr. Albert Fornace, Research Medical Officer, joined the Laboratory of Molecular Pharmacology, transferring from the Radiation Oncol. Branch, COP.

- Dr. Murray Munro, a Visiting Scientist in the Laboratory of Drug Discovery Research and Development, returned to the Univ. of Canterbury, Christchurch, New Zealand.

- Dr. John Blunt joined the Laboratory of Drug Discovery Research and Development as a Visiting Scientist; he comes to DCT from the University of Canterbury, Christchurch, New Zealand.

- Dr. Louis Malspeis was recruited as Head, Analytical Chemistry, Pharmacokinetics and Metabolism Section in the Laboratory of Drug Discovery Research and Development; he was formerly with Ohio State University.

- Dr. Ravi Varma was recruited as a Chemist in the Drug Synthesis and Chemistry Branch; he was formerly with E. R. Squibb and Sons.

- Dr. August Chiausa was recruited as a Cancer Expert in the Information Technology Branch; he was formerly with the Atlantic Research Corporation.

- Dr. David Segal was recruited as a Cancer Expert in the Information Technology Branch; he was formerly with Program Resources, Inc.

- Dr. John Beutler was recruited as a Cancer Expert in the Laboratory of Drug Discovery Research and Development; he was formerly with Program Resources, Inc.

- Dr. John Strong, Pharmacologist, Laboratory of Biological Chemistry, left to accept a position with the Food and Drug Administration.

- Dr. Richard Donovick, Cancer Expert with DTP, died on January 28, 1990.

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. In addition to the five branches in the COP, a laboratory under the supervision of Dr. Samuel Broder (Director, NCI) operates under the Office of the Associate Director. This Office also supports a Biostatists Data Management Section, supervised by Dr. Seth Steinberg.

Office of the Associate Director

A. Accomplishments in Antiretroviral Research

During the past year, new studies of AZT have been undertaken. One of the primary toxicities of AZT is bone marrow suppression. In an attempt to obviate this problem, a feasibility study was initiated to test whether AZT's bone marrow suppressive activity could be ameliorated by the administration of GM-CSF, a bone marrow stimulant that can promote the regeneration of cells belonging to the granulocyte and macrophage series. AZT was initially given in an alternating manner with GM-CSF to patients with AIDS. Ten patients were studied extensively, and the results suggest (1) that GM-CSF can potentiate bone marrow function in AIDS patients, and (2) that a regimen of GM-CSF and AZT is clinically active against human immunodeficiency virus (HIV).

The program has continued a feasibility study of AZT alternating with dideoxycytidine (ddC), a dideoxynucleoside, in patients with AIDS or AIDS-related complex (ARC). Some patients have been enrolled in this regimen for up to three years without significant toxicities. The combination of AZT and ddC seems to have reduced the level of bone marrow suppression observed with the administration of only oral AZT. The risk of peripheral neuropathy associated with single-agent ddC at high-dose continuous administration also seems to have been reduced significantly. The patients who developed a peripheral neuropathy usually had a mild, reversible form. Generally the peripheral neuropathy did not occur during the first six months of the regimen. These results have led to the initiation of a large-scale, multi-center study to determine whether an alternating regimen of AZT and ddC is more active and less toxic than single-agent therapy.

In July 1988 the program initiated a Phase I study of dideoxyinosine (ddI) in patients with AIDS or AIDS-related complex. This study demonstrated that at doses that were well tolerated patients had increases in T4 cells and total lymphocytes, decreases in HIV p24 antigen (a measure of the viral load), and other evidence of immunologic, virologic and clinical improvement. In addition, some patients had a reversal of HIV-dementia. At very high doses the limiting toxicities of ddI were found to be painful peripheral neuropathy, occasional pancreatitis, and occasionsl hepatitis. Doses of 200-750 mg/day of ddI, however, are associated with activity but rarely toxicity, and these are the doses being used in the Phase II/III trials. As a result of this Phase I study (with supporting data from two other studies), three Phase II/III studies of ddI were initiated in October 1990. In addition, ddI is being made available to patients who cannot tolerate AZT or have deteriorating disease while on AZT under the regulatory mechanisms of a "treatment IND" and "Expanded Access Program." This program has also observed that the survival of patients receiving ddI is quite good; overall, the survival of their patients with AIDS or severe AIDS-related complex was 88% at 20 months.

Medicine Branch

Accomplishments in Cancer Research:

Researchers in the Medicine Branch continue to gain experience with suramin in the treatment of prostate cancer. In men with hormone-refractory prostate cancer, suramin has produced objective tumor regression in 6 of 15 men with measurable

soft tissue components of their disease, a response rate of 40%. Not only is this one of the highest response rates ever achieved in this setting, but survival in this group appears to be significantly longer than that reported for any other salvage regimen. Recently pentosan, another agent that appears to block the binding of growth factors to cancer cells in vitro, entered Phase I clinical testing in the Medicine Branch.

Synthesis of thymidylate synthase (TS) is regulated at the level of translation by the amount of active enzyme. Thus, treatment with MTX or 5-FU results in the synthesis of new enzyme. Since inhibition of TS is important to the action of 5-FU, new synthesis of TS may significantly limit the action of 5-FU. Medicine Branch investigators have demonstrated that gamma-interferon can block this increase in TS at the level of translation. In the process of these studies, provocative information has been obtained on the ability of functional TS to cause feedback inhibition of synthesis of new enzyme by a translational block. A clinical trial in GI malignancies is testing the activity of drug-biologic combinations.

Taxol is a unique diterpene compound, isolated from the bark of the western yew tree, with significant activity against recurrent ovarian cancer. Investigators in the Medicine Branch are conducting a Phase I trial of taxol plus granulocyte colony-stimulating factor (G-CSF) to determine whether G-CSF, by protecting against granulocytopenia, can allow a higher dose of taxol to be administered safely and whether this higher dose is associated with greater antitumor activity. Early results suggest that roughly twice the standard dose of taxol can be safely administered with G-CSF bone marrow protection. G-CSF does not appear to be interfering with the antitumor activity of taxol, and several early responses have been seen.

NCI-Navy Medical Oncology Branch

Accomplishments in Cancer Research:

Chromosome and restriction fragment-length polymorphism studies have identified lesions on multiple chromosomes including chromosome regions 3p, 1p, 1q, 5q, 11p and 22. Because such tumor suppressor genes usually require inactivation of both the maternal and paternal chromosomes, this would indicate that as many as 10-15 different genetic lesions may be required for the development of clinically evident lung cancer. These results have direct bearing on future prevention and prognostic studies and may help direct the search for early molecular events in the detection of lung cancer and/or the detection of patients exhibiting some of these abnormalities in a premalignant phase. Laboratory studies are ongoing to try to "correct" these genetic abnormalities in lung cancer cells by reintroducing the suppressor genes into lung cancer cells through transfection and retroviral vectors.

High-pressure liquid chromatography studies of atrial natriuretic peptide in small cell lung cancer specimens and cell lines have shown that the peptide is similar to that secreted into the plasma by the right atrium. The prospective studies of ectopic production of atrial natriuretic peptide in small cell and non-small cell lung cancer patients are under way. These studies have potentially identified the natriuretic factor previously described in the syndrome of inappropriate

antidiuretic hormone (SIADH) present in patients with small cell lung cancer. These studies have led to a new understanding of the pathogenesis of SIADH and may lead to a new approach to treating hyponatremia in patients with small cell lung cancer.

The concurrent treatment of limited-stage small cell lung cancer using twice-daily chest radiotherapy and etoposide/cisplatin has approximately doubled the median survival of patients from approximately 15 months for historical controls to 28 months in a pilot Phase II study conducted by the NCI-Navy Medical Oncology Branch. More than 50% of patients are alive at two years. The results of this study have been adopted in cooperative group trials that are currently under way.

Pediatric Branch

Accomplishments in Cancer Research:

Protocol NCI 77.02-CCG 191 (Newly diagnosed patients with acute lymphoblastic leukemia) tested, in a randomized study, whether CNS preventive therapy using systemic high-dose methotrexate infusions alone (without cranial radiation) is equally effective and less toxic than 2400 cGy of cranial radiation and intrathecal methotrexate. The overall remission rate was 98% with an event-free survival of approximately 70% at three years from the entire study group. With a median duration on study of 76 months, there is no significant difference in the CNS relapse rate for either treatment group. A recent analysis of patients longitudinally assessed with a periodic neurophyschological test battery demonstrated a striking decrease in verbal and full-scale IQ in patients treated with cranial irradiation and intrathecal chemotherapy. In addition, patients treated with cranial radiation and intrathecal therapy manifested significant impairment of academic achievement. No such declines were seen in the high-dose methotrexate groups. These data thus indicate that use of combined cranial radiation and intrathecal therapy can be avoided in nearly 60% of children with ALL, reducing the potential long-term neurotoxicity associated with such combined therapy. In contrast, this study has demonstrated no apparent adverse effects of high-dose methotrexate on cognitive functioning and academic achievement, confirming the value of high-dose methotrexate as CNS preventive therapy for children with ALL.

Lymphoblasts from 28 patients were studied for evidence of *mdr*-1/P-170, the gene encoding for the plasma membrane glycoprotein associated with multidrug resistance, using RNase protection, RNA *in situ* hybridization and immunochemistry. Overexpression with gene amplification was identified in the cells of three relapsed patients and from one patient at diagnosis (this patient failed to achieve a completed remission with induction therapy). *In situ* hybridization, immunochemistry and drug-uptake studies demonstrate that this overexpression is heterogeneous. It appears from these studies that overexpression of *mdr*-1/P-170 is one mechanism of drug resistance in ALL. A protocol for relapsed ALL patients who express the MDR phenotype is being initiated to attempt to reverse this form of drug resistance.

Pediatric Branch investigators have been able to specifically inhibit expression of the c-*myc* gene in a subset of Burkitt's lymphomas. This has been accomplished by using an antisense oligonucleotide directed against intron sequences that are present in the mature messenger RNA species in Burkitt's lymphomas with c-*myc* first intron breakpoints on chromosome 8. Both cellular proliferation and c-*myc*

protein expression were inhibited in the experiments. These findings demonstrate that the molecular abnormalities in tumors may also provide a target for specific therapeutic endeavors. Because only Burkitt's lymphoma cells, and not normal cells, contain the genetic abnormalities, such therapeutic approaches may be highly selective. The Branch is pursuing preclinical studies with antisense oligonucleotides using Burkitt's lymphoma xenografts in a nude-mouse model.

Investigators in the Pediatric Branch have developed a laboratory model of candidiasis to evaluate promising antifungal agents. These include models for acute disseminated infection, chronic infection (e.g., hepatosplenic candidiasis), subacute or local infection and phrophylaxis. This permits a more reliable assessment of antifungal strategies and suggests that a new triazole, fluconazole, may offer benefit for early (e.g., prophylactic) use in neutropenic hosts. Accordingly, a randomized clinical trial to assess the utility of prophylactic fluconazole in pediatric and adult cancer patients is planned to begin in 1991.

Accomplishments in Antiretroviral Research:

In a search for an effective, less toxic (than AZT) regimen for children with symptomatic HIV infection, the Pediatric Branch initiated a Phase I-II trial of dideoxyinosine (ddI) in children in January 1989. To date, 78 children have been enrolled at several dosage levels. The protocol enrolls both children who have received no prior antiretroviral therapy and children who have become refractory or intolerant to AZT. ddI was rapidly absorbed after oral administration; however, there was significant variability in its bioavailability. Pancreatitis occurred in two patients, one at each of the two highest dose levels. Median CD4 cell count increased from 218/mm^3 at baseline to 327/mm^3 at 24 weeks ($p=0.001$). Patients with baseline CD4 cell counts greater than 100/mm^3 were significantly more likely to show an increase in this parameter. Median p24 antigen declined from baseline to 24 weeks ($p=0.005$) and there was a significant correlation between ddI plasma concentration and decline in p24 antigen level. A significant correlation was also found between ddI plasma concentration and improvement in cognitive function. Improvements in clinical and immunological parameters occurred in previously untreated patients and in prior AZT recipients. Dideoxyinosine was well tolerated and shows promising antiretroviral activity in HIV-infected children. The correlation between response and plasma ddI concentration indicates that bioavailability is an essential consideration for optimizing ddI activity in the treatment of HIV infection.

Radiation Oncology Branch

Accomplishments in Cancer Research:

ROB scientists have utilized hematoporphyrin derivative and laser-controlled sources of light in the treatment of superficial cancers of the skin and mucous membranes, so-called photodynamic therapy (PDT). They have also used this technique to reopen occluded bronchi and to treat peritoneal surfaces by means of intracavitary administration of the light. The preliminary results on the peritoneal cases with ovarian cancer appear quite promising as part of a Phase I study in patients with recurrent disease. In addition to peritoneal investigation, the ROB has begun to investigate the role of photodynamic therapy of the pleura with a Phase I study that is focusing on mesothelioma.

In the laboratory, the ROB continues to focus on the mechanisms of radiation sensitization, protection and resistance. They have studied sulfhydryl compounds, especially glutathione, and its relationship to cell killing or protection by either radiation or chemotherapy. Additional work has gone on in heat shock proteins and in the characterization of human tumor cell lines in conjunction with other Branches. The laboratory has demonstrated conclusively that cells that are pleiotropically drug-resistant are not necessarily resistant to radiation therapy; moreover, such cells are definitely not resistant to photodynamic therapy.

Surgery Branch

Accomplishments in Cancer Research:

Significant therapeutic synergies have been seen in animal models using tumor-infiltrating lymphocytes (TIL) in combination with local radiation therapy or alpha-interferon. These combined treatments utilizing TIL can result in the elimination of tumor burdens greater than those successfully treated by TIL and interleukin-2 (IL-2) alone. These studies have important implications for the treatment of human cancer.

Genes coding for tumor necrosis factor have been successfully introduced into human TILs. These TILs produce over 100 times the normal level of tumor necrosis factor (TNF) and have significant concentrations of membrane-bound TNF as well. Plans are under way to utilize these TNF-modified enhanced TILs for the treatment of human cancer.

Using both mouse and human TILs, the Surgery Branch has shown that cytokines can be specifically released from TIL populations when stimulated by the tumor. This technique represents an alternate method for identifying specific immune reactions in patients with cancer. The cytokines produced by human and murine TILs include gamma-interferon, GM-CSF, TNF-alpha and IL-3.

Significant therapeutic synergies have been seen in animal models using combination cytokine treatment. The most effective combinations studied are IL-2 and alpha-interferon as well as the use of IL-2 and TNF. Recently the combined administration of IL-2, TNF and alpha-interferon has been shown to be more effective than any of the two cytokines alone. These studies are forming the foundation for combination cytokine therapy in man.

Polymorphic probes localized to the short arm of chromosome 3 have been used to detect the loss of heterozygosity at this locus in tumor tissue from 51 of 58 patients with sporadic renal cell carcinoma. Deletion analysis has shown that this is in the same region as the gene for the familial form of renal cell carcinoma associated with Von Hillel Lindau disease.

Studies of the molecular genetics of renal cell cancer have demonstrated abnormalities at recessive gene loci on chromosome 11 near the Wilms locus, on chromosome 13 at the retinoblastoma locus, and on chromosome 17 at the NM 23 locus. The presence of these abnormalities from patients with advanced renal cancer suggests that loss of these functional genes may be associated with progression or metastases of renal cell cancer.

The first gene transfer trials conducted in man have begun. Eight patients with advanced melanoma have received treatment with autologous TIL modified with the gene coding for neomycin phosphotransferase that confers resistance to the antibiotic neomycin. These studies have shown that gene-modified cells can survive for up to 189 days in the circulation and for up to 64 days at the tumor site. No toxicity has been seen in patients due to the gene transfer. Clinical trials utilizing TIL modified by the gene for TNF are planned.

A combination regimen of 5-FU and leucovorin and IL-2 has undergone Phase I and II testing. This regimen has shown a 55% incidence of complete and partial responses in patients with metastatic colon cancer and is undergoing an evaluation of predictors and correlates of response.

RADIATION RESEARCH PROGRAM (Associate Director, John Antoine)

The Radiation Research Program (RRP) is an extramural program having two branches: the Diagnostic Imaging Research Branch (DIRB), and the Radiotherapy Development Branch (RDB). The scientific mission of the RRP is to develop research programs for the extramural community in which radiation and related forms of energy are used in the diagnosis, staging, treatment and post-treatment evaluation of the patient with cancer.

Accomplishments in Cancer Research:

A high-priority research area of the RRP is and has been the Fast Neutron Clinical Trials project. Phase III trials have been designed and are successfully being carried out in this project. The institutions participating in these trials continue to be the University of Washington, Seattle; UCLA, Los Angeles; and the University of Texas Cancer Center M.D. Anderson Hospital, Houston. The efficacy of neutron beam therapy for the treatment of malignant salivary gland tumors has been demonstrated. Data continue to be obtained from clinical trials in the treatment of localized prostate cancer, head and neck tumors and radio-resistant neoplasms. It is estimated the trials can be concluded by 1992-93.

Multi-institutional clinical trials in diagnostic radiology are conducted by the Radiologic Diagnostic Oncology Group (RDOG). The objectives of the trials are to use single or multiple new imaging technologies to diagnose, stage and monitor cancers and to develop an alogrithm for the appropriate sequential, cost-effective selection of diagnostic procedures. Data from the first trial, RDOG 1, on lung and prostate cancer suggest that MRI is not significantly better than CT in establishing extent of mediastinal involvement by lung cancer and that neither MRI nor ultrasound is sensitive enough to replace surgery in determining extent of locoregional spread of early-stage prognostic cancer. RDOG 2, now in progress, is examining the diagnosis, staging and monitoring of colorectal and pancreatic tumors. The third trial will study musculoskeletal and head and neck tumors.

Radiation sensitizers continue to be investigated for use with radiation therapy. SR-2508 is currently in Phase III clinical trials in Europe and the United States, with the investigation in the U.S. being carried out by the Radiation Therapy Oncology Group (RTOG). Other notable compounds identified by the radiation sensitizer contrats of RRP included BSO and nicotinamide. SR-4233 appears to be not only a radiation sensitizer but also a selective hypoxic cell cytotoxic agent.

Fast neutron therapy is clearly successful in the treatment of malignant salivary gland tumors. There appears to be a significant advantage (20%) of fast neutrons over photons in Stages C and D1 prostate cancer. There also appears to be some promise in the tretment of advanced rectal cancers by use of the fast neutron beam.

DEVELOPMENTAL THERAPEUTICS PROGRAM (Acting Associate Director, Michael Grever)

The Developmental Therapeutics Program (DTP) is the preclinical unit within the DCT charged with the discovery and development of new anticancer and anti-HIV agents for introduction into clinical trials. The DTP utilizes both intramural and extramural (grant and contract) mechanisms to accomplish its mission.

A new intramural unit, the Laboratory of Drug Discovery Research and Development, was established for the expeditious development of high-priority agents for the treatment of either cancer or HIV infection. In this sense, the laboratory represents a combination of both applied and basic research capabilities that can be rapidly mobilized to address problems that might delay a promising drug getting to clinical trial. In addition, the laboratory will maximize the drug discovery and development processes already operational within DTP. Finally, the natural-product drug discovery effort represents another major focus for investigators within this laboratory.

A. Accomplishments in Cancer Research:

Development and Implementation of Disease-Oriented Anticancer & Anti-HIV Screens

The current anticancer screen in the Biological Testing Branch (BTB) has reached an annual capacity of 15,000 tests per year with an expectation to reach the 20,000 annual capacity very soon. Prostate and breast cancer cell lines for screening are under development. Protocols for secondary in vitro confirmatory testing have been developed, and approximately 90% of the current in vitro panel lines are available for subsequent in vivo testing. The EORTC agreements are in place for in vivo studies for supplementing and confirming of results.

In the BTB, the AIDS in vitro screen is operation at a level exceeding 40,000 tests annually, with an expected annual test capability of 50,000 tests. Confirmatory testing of active agents includes a syncitium assay and the production of HIV p24 antigen. Currently available in vivo models have limited application for the disease in man; therefore, model development continues with several potential candidate models, including a closely related lentivirus (BIV) and the SCID/NIH III mouse carrying HIV.

Discovery and Development of Anticancer Agents

A new approach to the developmental process for anti-HIV and anticancer agents has been instituted. Those agents that are identified as being high-priority are assigned to a specific individual within DTP, and a special working group of interdisciplinary investigators is assigned to each project. In contrast to the special working groups that were informally convened by DTP staff in the past, the current working groups must meet at regular intervals and provide progress reports to the Associate Director at bi-monthly intervals. Furthermore, the chairperson for each group must be responsible for the agent until it formally enters Phase I clinical trial in patients.

In Vitro Screening of Anticancer and Anti-HIV Compounds

For the past year the anticancer and anti-HIV screens, which use a variety of human cell lines to test promising new compounds, have been fully operational. This screen is now operating at approximately 300-400 tests per week and is working through the backlog of compounds with known chemical structure that have accumulated over the period of time that the screen was in preparation. Since April there have been over 3,000 reports mailed to new compound sponsors. There have been 46 compounds that have demonstrated sufficient activity in the screen to be recommended for further testing. As the backlog of submitted structures is reduced, screening of natural-product extracts will resume. This broad-based approach to the systematic testing of natural and synthetic compounds will optimize the chances for identification of effective new agents to treat cancer and AIDS.

Extension of Program Capabilities

As a complement to the anticancer screening effort, DTP established a National Cooperative Drug Discovery Group (NCDDG) program in 1983. This effort exploits recent developments in biomedical research for the discovery of innovative treatment for cancer. Multidisciplinary and multi-institutional teams of the nation's most talented scientists from academic, nonprofit research and commercial organizations are brought together to conceive and develop new drug and treatment strategies utilizing novel modes that will accurately predict the efficacy of research efforts. Currently there are 11 funded groups and 12 more have been approved for funding. Approximately $15 million is earmarked for this important aspect of the program. These new awards will greatly expand efforts to discover new anticancer agents from natural sources, such as the tropical rain forests and marine habitats, and will stimulate diverse types of research projects to find new therapies using three general approaches: general mechanism of action, specific disease-oriented strategy, and novel model development.

Taxol

Increasing the supply of taxol has become one of the highest priorities of the NCI. Recognition that this unique compound has significant activity in refractory malignancies, such as ovarian and breast cancers, has stimulated a search for means by which taxol may be made more readily available. Currently, taxol must be extracted from the bark of mature western yew trees. Yew trees are found only in a limited sectin of the Pacific Northwest and removal of the bark requires the sacrifice of the tree. The 5-10 pounds of bark obtained in this process yield enough taxol for a single therapeutic treatment. Obviously, this process has severe limitations and will be useful only for the short-term supply of drug. This problem in being addressed in a number of ways. In July, the DTP issued a Request for Applications (RFA) for chemical and/or biological studies of taxol that could ultimately lead to ways of increasing production or further define its biological properties for use in improved drug design or in enhanced clinical utility. It is expected that this RFA will be funded beginning in 1991. The NCI is also negotiating a Collaborative Research and Development Agreement with Bristol-Myers Squibb that will add the considerable resources of this major pharmaceutical company to address the challenge of obtaining adequate supplies of taxol from the bark of the western yew tree. And lastly, DTP is supporting a number of researchers who are

investigating the semisynthesis of taxol from a renewable resource, such as the leaves of the yew tree, or the identification of other compounds that work in the same way as taxol to prevent neoplastic growth through stabilization of microtubule formation. These efforts will be closely monitored and coordinated out of the Office of the Director, DCT, and the Office of the Director, NCI.

DNA Damage and Repair

Studies of preferential DNA repair in active genes have been undertaken by the Laboratory of Molecular Pharmacology (LMP). Several important new observations include (a) nitrogen mustard adducts form to differing extents in different genomic regions and are repaired preferentially; (b) in a comparison of two human ovarian cancer cell lines of differing drug sensitivity, the cisplatin-induced adduct repair in the DHFR gene was similar in the two lines, but the DNA interstrand crosslink removal was slower in the sensitive line; (c) UV-induced damage was more efficiently repaired in a resistant cell subset from a panel of plasmacytoma cell lines; and (d) gene-specific repair of UV-induced damage was inhibited in CHO cells in the presence of inhibitors of both topoisomerase-I and merbarone (reported to inhibit topoisomerase without inducing cleavage complexes). The LMB is currently exploring the role of gene-specific repair in multidrug resistance.

The LMP is also working on the isolation and characterization of genes whose expression is induced by cell injury by agents such as ultraviolet light, heat and chemotherapeutic agents. The mechanism of subsequent cell death following the induction of genes responsive to DNA damage is being explored. Twenty different cDNA transcripts induced by DNA damage have been isolated and sequenced. In addition, a large collection of mammalian cDNA clones for heat-shock genes have been isolated.

GTP-Binding Proteins

The emerging family of structurally related proteins (i.e., small GTP-binding proteins) has been implicated as regulators of a diverse array of important cellular functions, including: cell transformation and growth, protein synthesis and processing, targeting of membrane vesicles and organelles, and activation of secondary messenger systems (e.g., phospholipase C). While all members of this class of proteins may share similar mechanisms of cellular regulation, the Laboratory of Biological Chemistry (LBC) has focused on the ARF proteins (i.e., ADP-ribosylation factors). The ARF proteins in mammalian cells are critically important for protein secretion, and the role of ARF in regulation of growth factors is being actively pursued. Specific functional domains of the ARF proteins are being mapped with both point and deletion mutations as well as antibody probes. Peptide inhibitors of ARF have been constructed and are being investigated in vitro to determine the role of ARF in protein secretion. These efforts could eventually result in new targets for chemotherapeutic intervention.

Discovery of Novel Leads for Drug Development Against Cancer and AIDS

During the past year the Drug Synthesis and Chemistry Branch (DS&CB) acquired approximately 7,000 new compounds from 677 suppliers (280 pure natural products, 6720 synthetic compounds). The DS&CB plays a key role in operation of the Acquisition Input Committee for both the cancer and AIDS screens. Approximately 200 compounds are tested weekly in the AIDS screen, and there are 300 compounds tested weekly in the cancer screen.

Active leads are optimized through synthesis of prodrugs and congeners. Current DS&CB efforts include synthesis of ellipticinium analogs, castanospermine analogs, diazo dyes, active ATA fractions, novel platinum analogs and bombesin antagonists.

Studies on Hazards of New Investigational Agents to Healthy Organs in Intact Experimental Animals

Research projects in the Toxicology Branch over the past year have focused on innovative methods to compare metabolism between species to project both toxicologic and efficacy differences based on metabolism. A project has been launched to compare the metabolism of anti-HIV agents in lymphocytes from experimental species and humans. An attempt is also being made to compare in the AIDS screen the antiviral activity of heat-inactivated serum samples taken from animals following in vivo treatment with the anti-HIV agent and an in vitro direct exposure to the same drug. This type of investigation may permit an assessment of the antiviral activity of a specific agent in vivo.

Grants and Contracts Operation Branch (G&COB)

Although several important research findings were made over the past year, three grantees made outstanding contributions. Dr. Stephen Lippard's team at the Massachusetts Institute of Technology isolated cDNAs modified by the anticancer drug cisplatin. These investigations may shed light on the mechanism of tumor cell repair of cisplatin-induced DNA damage. Dr. Igor Roninson's team at the University of Illinois demonstrated a more sensitive method for measuring multidrug resistance utilizing the polymerase chain reaction, and subsequently demonstrated that human tumor cells have a non-uniform distribution of this property. Dr. Monroe Wall improved the synthesis for 9-amino-20(RS)-camptothecin, an agent selected for clinical development by the NCI based on encouraging preclinical antitumor data.

B. Accomplishments in Antiretroviral Research:

Antiviral Evaluations Effort

The Antiviral Evaluations Branch provides an effective unit for the screening and further evaluation of anti-HIV drugs. Standardization and further refinement of the primary in vitro anti-HIV drug screen continued, and the large-scale screening capacity progressively increased. As of May 1990, approximately 90,000 screening tests had been performed, and over 16,000 unique synthetic compounds and 15,000 extracts from biological materials had been tested. In addition, over 6,000 fractions from active biological extracts had been further examined. From these tests, approximately 250 pure compounds and 300 extracts had been found to be active. Among the active pure compounds, new members of seven chemical classes already-known to be active against HIV were represented. More significantly, new active lead compounds and related derivatives have been discovered in 14 chemical classes not heretofore associated with anti-HIV activity. Development and implementation of confirmatory tests for anti-HIV activity, i.e., testing for inhibition of virus production as well as inhibition of virus-induced cell killing, have become standard. Also, protocols that allow testing of active compounds for range of action and determination of mechanism of action have been implemented. In addition to a compound's activity in the primary screen, testing in other cell lines

and against other viruses (e.g., HIV2) are performed. Collaborative experiments focusing on the stage of virus reproduction affected are under way. These latter studies are designed to provide valuable information relevant to the development of the compound for eventual clinical use.

Discovery and Development of New Anticancer and Antiviral Drugs

The Laboratory of Medicinal Chemistry's (LMC) development and improvement in synthesis for the fluorodideoxynucleosides will potentially benefit the anti-HIV drug discovery and development program. Demonstration of acid stability suggests that an oral formulation may be appropriate.

LMC work continues on the development of inhibitors of both protein kinase C and tyrosine kinase. The most likely conformation of diacylglycerol has been established for its binding to the regulatory site of the PKC. Of interest, a compound has been identified that inhibits the autophosphorylation of the tyrosine kinase without affecting exogenous substrate phosphorylation.

Computerized molecular modeling techniques are currently directed to addressing the following LMC projects: 1) design of competitive inhibitors of PKC, 2) design of dideoxynucleosides based on active members of the series, and 3) quantitative structure-activity relationships on inhibitors of tyrosine kinase.

Over the past year, the Laboratory of Biochemical Pharmacology (which was abolished in August 1990 and staff incorporated into the Laboratory of Medicinal Chemistry) has investigated the cellular pharmacology of 2',3'-dideoxy-2'-fluoro-arabinosyladenine (fddA) in several cell lines. This agent is acid-resistant and thus is potentially amenable to oral administration. fddA is more resistant to deamination than ddA, and anabolism to the diphosphate and the triphosphate is more energetic in comparison to ddA.

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

The Biologic Response Modifiers Program (BRMP) is a comprehensive program with both intramural and extramural basic and clinical research components charged with investigation, development and clinical testing of biological approaches to cancer treatment.

A. Accomplishments in Cancer Research:

Dr. Jonathan Ashwell and colleagues have been the first to recognize that each T lymphocyte has two types of T-cell receptors. While the extracellular domains are similar, each receptor has very distinct signal transduction pathways. The relative expression of each receptor may influence the ability of the cell to recognize or become tolerant to antigen presentation, a process essential for the development of a competent immune system.

The thymus gland is felt to play a critical role in the development of T lymphocytes. Dr. Ada Kruisbeek has been investigating the roles of cytokines IL-2 and IL-4 in early T-cell development before the cells express T-cell antigen receptors. It appears that once surface receptors are expressed, the ultimate fate of the clone depends on complex interactions between the T-cell receptor, CD4 and CD8

antigens on the lymphocyte, and Class I and II major histocompatability complex molecules on the thymic epithelial or stromal tissue. An in vitro model of the thymus gland is being developed to examine this complex system more precisely. Insight into the regulation of T cells can prove quite valuable in enhancing organ transplantation safety.

In the Laboratory of Molecular Immunoregulation, Dr. Joost Oppenheim has examined the effect of IL-1 in mice exposed to lethal doses of radiation. He has demonstrated that IL-1 improves the capacity of limited numbers of cells from the bone marrow to repopulate the recipient. Therefore, pretreatment of animals with IL-1 may protect the marrow against the lethal effects of radiation and may hasten the reconstitution of bone marrow following radiation therapy. IL-1 thus appears to be both a radioprotector and a colony-stimulating factor.

B. Accomplishments in Antiretroviral Research:

In the Laboratory of Molecular Immunoregulation, Dr. Frank Ruscetti and colleagues are examining the mechanism by which cells regulate the expression of human retrovirus, HTLV-1 and HIV. Investigators have found that cells infected with HTLV-I produce a factor for the transactivation of viral replication. Efforts are under way to identify this regulation of viral expression. This may have important implications for the well known latency of HIV infection, especially in monocytes. Demethylation with 5-azacytidine is one form of this latency. Other forms of latency appear to exist and may be due to nuclear factors that are produced by the cell. Further studies are planned to identify these important regulatory proteins.

Dr. Hsaing-Fu Kung of the Laboratory of Biochemical Physiology has pursued this issue in HIV-infected monocytes. Dr. Kung and colleagues have isolated genes for several previously unappreciated DNA binding proteins that bind to the HIV long terminal repeat (LTR) at specific sequences that appear to inhibit HIV replication. Further characterization of these genes and their protein products will provide information that will be vital to gaining further insight into the life cycle of HIV as well as into the development of therapies that may be effective against cells containing dormant HIV.

CANCER TREATMENT EVALUATION PROGRAM (Associate Director, Michael Friedman)

The Cancer Therapy 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 holders of the investigator-initiated grants (R01 and P01) relating to cancer treatment.

A. Accomplishments in Cancer Research:

Consensus Development Conferences

Based on considerable trial data generated by the Cooperative Groups, two cancer therapy-related Consensus Development Conferences were sponsored by the Office of Medical Applications of Research at the National Institutes of Health. One dealt with adjuvant therapy for large-bowel cancer patients. It endorsed the use

of postoperative pelvic irradiation and systemic 5-FU and levamisole for Stage III colon cancer patients. For the first time, a consensus was possible on this subject. The other conference confirmed the value of less extensive surgery for early-stage breast cancer and adjuvant systemic therapy for poor-prognosis node-negative patients.

High-Priority Clinical Trials

Figures obtained by CTEP show that accrual to the High-Priority Trials continues to accelerate, and the rate of entry of new patients onto these studies is well beyond the average rate of accrual to other large Phase III treatment studies. The ten active High-Priority Trials now are accruing patients about three times the average rate of the other Phase III trials.

Five Phase III studies originally were identified as High-Priority Trials (Series I). Four of the five trials have succeeded in accruing patients more rapidly than initially anticipated. The National Surgical Adjuvant Breast and Bowel Project (NSABP) Study C-03 reached its accrual goal and closed April 1989. The North Central Cancer Treatment Group (NCCTG) rectal study (864751) is accruing at three times the initially projected rate and will close soon (more than 1.5 years earlier than anticipated); its replacement study is being prepared. The NSABP rectal study (NSABP-R02) and Southwest Oncology Group (SWOG) lymphoma intergroup (INT-0067) trial are entering patients somewhat more quickly than planned and are expected to complete accrual on or ahead of schedule. The SWOG Bladder intergroup study is an unprecedented intergroup effort (INT-0080) and continues to accrue below the planned rate.

In June 1989 six additional trials were approved by the Chairman of NCI's Clinical Trials Cooperative Groups and received the High-Priority designation (Series II). Although these are mostly very large studies, their planned accrual periods are all less than 3.3 years. Accruing beyond their projected rates after approximately one year are the Eastern Cooperative Oncology Group small cell lung cancer study (INT-0096) and intergroup node-negative breast cancer study (INT-0102). These studies will complete accrual in less than the projected time period if the current rates continue. The Radiation Therapy Oncology Group lung intergroup study (RTOG-8808), which opened in January 1989, also is accruing patients more quickly than anticipated.

The NSABP breast study B-18 addresses an adjuvant population and is accruing at nearly the projected rate, while the NSABP breast study B-21 (Occult Stage I Disease) deals with a unique patient population and is entering patients more slowly than planned. The further study of levamisole in colon cancer (INT-0089) is accruing patients very rapidly and should be completed ahead of schedule.

Laboratory-Clinical Correlations

Over the past few years, the Clinical Trials Cooperative Groups have become increasingly interested in integrating important laboratory science into clinical trials. Intergroup studies in breast cancer have been evaluated and are continuing to incorporate analyses of a number of potential prognostic factors in adjuvant trials, including HER-2/neu oncogene expression as well as flow cytometry and cathepsin D, S-phase fraction, haptoglobin-related protein, and estrogen receptor determination by immunochtochemistry.

Pediatric neuroblastoma studies are currently stratifying patients on the basis of n-myc amplification.

SWOG is planning to evaluate specimens from patients with ovarian cancer for the multidrug-resistance phenotype and glutathione-S-transferase to develop approaches to circumvent acquired drug resistance.

The Cancer and Leukemia Group B (CALGB) has established a series of companion studies to the colon adjuvant trials that will assess the importance of molecular genetic changes, laminin binding proteins, and the clinical significance of tumor progression genes. The Eastern Cooperative Oncology Group (ECOG) and NCCTG are evaluating the prognostic implications of ploidy and proliferative activity in patients with primary colorectal carcinoma.

B. Disease-Specific Accomplishments:

Testis Cancer

The ECOG conducted an interim analysis of their trial comparing bleomycin, etoposide and platinum (BEP) versus etoposide and cisplatin for patients with minimal- or moderate-risk metastatic disease by Indiana criteria. This study was designed as a toxicity-reduction trial, hoping to avoid the toxicities of bleomycin. The relapse rate was found to be significantly higher in the patients receiving the 2-drug regimen for three cycles compared to the 3-drug regimen for three cycles. The study has thus been closed with the recommendation that three cycles of BEP should continue to be the standard treatment for these patients.

Ovarian Carcinoma

Accomplishments. Two important Phase III trials in advanced sub-optimally debulked ovarian cancer trials have recently been completed. The SWOG demonstrated that the combination of CBDCA and cyclophosphamide had efficacy similar to cisplatin and cyclophosphamide. However, women receiving CBDCA and cyclophosphamide experienced substantially less nausea, vomiting and neurotoxicity. The GOG randomized women to two different cisplatin-cyclophosphamide regimens differing in dose-intensity. The results of this study will help to define the importance of dose-intensity in ovarian cancer.

The population of patients with advanced (Stage III), optimally debulked ovarian tumors is the focus of a Phase III intergroup effort, evaluating intraperitoneal CDDP with systemic CYT versus intravenous CDDP/CYT. This is a very important trial, involving the SWOG and the Gynecologic Oncology Group (GOG), given the theoretical advantage of regional therapy in small-volume ovarian cancer and is a large enough study to conclusively determine the role of up-front intraperitoneal therapy in this group of patients.

Future plans. Taxol has emerged as the most active new cytotoxic agent against ovarian cancer in the last ten years. The GOG recently initiated a protocol comparing taxol and cisplatin to standard therapy (cyclophosphamide and cisplatin). Additionally, the GOG is defining the optimal intraperitoneal dose of taxol in a Phase I trial, while several systemic Phase I studies are attempting to determine the maximum dose of taxol that can be safely administered by combining it with a hematopoietic colony-stimulating factor.

Breast Cancer

Accomplishments. Two studies that were closed to accrual in recent years were analyzed this year and have demonstrated improved survival for different subgroups of patients treated with combination chemotherapy and tamoxifen. In EST-5181, premenopausal women with node-positive breast cancers were randomized to one of two chemotherapy combinations. At the completion of chemotherapy they were again randomized to either tamoxifen for five years or no further therapy. A recent interim analysis has found that tamoxifen in addition to chemotherapy prolongs the survival of patients with estrogen receptor-positive tumors. The recommendation for this group of patients has previously been that chemotherapy alone represents the standard of care. An ongoing intergroup study is designed such that this finding can be confirmed in the near future. Another study, NSABP B-16, was designed to compare standard tamoxifen therapy for postmenopausal women with hormone-responsive, node-positive breast cancer with tamoxifen plus combination chemotherapy. This study, reported in June 1990 in the JOURNAL OF CLINICAL ONCOLOGY, has found that treatment with adriamycin plus cyclophosphamide in addition to tamoxifen enhances the chance for survival compared to tamoxifen alone at a median of three years of follow-up. Both of these studies will require further follow-up for more conclusive interpretations (such that standard therapy might be changed).

Future plans. The role of high-dose chemotherapy with autologous bone marrow rescue given as adjuvant therapy for patients with very high risk of recurrence following local resection (Stage II or IIIa with >10 axillary lymph nodes) will be evaluated in a randomized Phase III trial. In order to design the best trial(s) possible, a strategy meeting will be held by CTEP in September 1990. This should not only identify the best possible current design, but should help identify additional development trials (role of IL-3, role and timing of peripheral blood stem cell harvesting) that need to be done.

The Cooperative Groups will be asked to help plan, coordinate and carry out a trial of tamoxifen as a chemopreventive agent for women over age 50. Because tamoxifen lowers serum cholesterol and blood lipids, a secondary end point will be incidence of cardiovascular morbidity.

The Cooperative Groups will be approached about contributing to the Cooperative Human Tissue Network (a tumor bank) so that there is a more available resource for accessing tumor samples in patients with clinical follow-up so new therapeutic and prognostic factors can be evaluated in a time-efficient manner.

Colony-Stimulating Factors

A great deal of information regarding the toxicity and effectiveness of granulocyte (G-CSF) and granulocyte macrophage (GM-CSF) colony-stimulating factors when used to reduce the side effects of chemotherapy or to allow higher doses of chemotherapy has now been developed. Both drugs appear to reduce the frequency of neutropenia following chemotherapy and, as a result, may reduce the frequency of neutropenic fever and infections. Little effect to counter the chemotherapy-induced thrombocytopenia has been observed, however, with either G-CSF or GM-CSF. Newer agents such as IL-1, IL-3 or IL-6 when used in conjunction with G-CSF or GM-CSF may provide protection against thrombocytopenia. Preliminary information has also implied that the CSFs might reduce the mucositis and diarrhea which occur after

some forms of chemotherapy. Clinical trials to definitely evaluate the true ability of the growth factors to moderate these toxicities are being designed.

Antigen-Directed Therapies

The binding characteristics, biological activities and pharmacokinetics of murine antibodies to a wide range of hematopoietic and solid tumor-associated antigens have been studied over the last several years. Some of the most interesting of these antibodies have been chimerized, and these unconjugated monoclonal antibodies, with their promise of longer circulating half-lives, reduced immunogenicity and increased immunobiological activity, are now being introduced into NCI-sponsored trials. Phase I trials have begun with one of these chimerics directed against the TAG-72 adenocarcinoma-associated antigen. At the same time, trials investigating the combination of monoclonal antibodies with immunostimulatory cytokines, including IL-2, interferons, GM-CSF and M-CSF, have been initiated.

Through a series of NCI-sponsored contracts, clinical trials are under way to establish optimal means of utilizing radioimmunoconjugates in therapy directed against adenocarcinomas. The antibody targets initially being studied include both the TAG-72 and CA antigens; radiolocalization and pharmacokinetic studies will compare the characteristics of radiolabeled monoclonal antibodies of different affinities, of antibody fragments as compared to whole antibody, and of chimeric antibody in relation to the parent murine antibody. The maximum-tolerated doses of these immunoconjugates administered as single doses, or alternatively in multiple dose fractions, are being determined. In other trials the relative characteristics of alternative isotopes, including Yttrium-90, are under study.

Differentiating Agents

Trans-retinoic acid (tRA) has recently been shown in separate Chinese and French trials to have activity in the treatment of acute promyelocytic leukemia (APL). The NCI will investigate tRA in a new oral formulation. Phase I trials to determine the maximum-tolerated dose of tRA (alone and in combination with other differentiating agents) are now under way. An Intergroup confirmatory Phase II trial of tRA activity in APL is currently in the design phase; a special exemption protocol for American APL patients requiring tRA (while the Phase I evaluations of this agent are being completed) is currently being implemented.

Mechanisms to Overcome Antimetabolite Resistance

Biochemical modulation involves the combination of inactive or minimally active compounds with chemotherapeutic agents that have established antitumor activity with agents that alter the cellular actions of the active drug in order to enhance its therapeutic effectiveness. 5-FU has been at the center of this research. The modulating compound may alter the drug's extracellular or intracellular metabolism, act as a cofactor in enzymatic reactions (e.g., leucovorin), inhibit critical enzymes (e.g., PALA or IFN-gamma), block transport systems (e.g., dipyridamole), replace nucleotides in DNA (e.g., AZT) or RNA, selectively rescue or protect normal tissues (e.g., uridine), or act through other mechanisms not yet determined (e.g., levamisole).

The combination of leucovorin and 5-FU has now become the standard regimen for patients with advanced colorectal adenocarcinoma. Leucovorin was the first biomedical modulator of 5-FU proven to have clinically relevant effects and is

being evaluated by the FDA for this indication. Many of the compounds referred to above are being evaluated in many different combinations and schedules, against different cancers, and both in advanced disease and in the adjuvant setting. Clinical trials using combinations of modulators (e.g., 5-FU/PALA/Dip or 5-FU/LV/PALA) are also under way. The most impressive results remain in the colorectal cancer patients. The most active combinations to date are 5-FU/PALA and 5-FU/IFN-gamma in advanced disease and 5-FU/levamisole in the adjuvant setting. Large multicenter Phase III studies to confirm the positive Phase II trials are active or planned.

Reversal of Resistance to Alkylating Agents

Intracellular elevations of glutathione (GSH) have also been shown to be associated with primary and acquired resistance in some experimental models of human cancer. Studies indicate that administration of buthionine sulfoxamine (BSO), a potent inhibitor of the first and rate-limiting step of GSH biosynthesis, to animals or cultured cells results in tissue and cellular GSH depletion, suggesting the potential for reversing resistance mechanisms associated with increased levels of GSH. The compound has been shown to reverse the acquired resistance of human ovarian cell lines to either melphalan and/or platinum. Clinical trials are ongoing to evaluate a combination of BSO together with L-PAM for the potential of reversing drug resistance. Preliminary data indicate that BSO induces in vivo depletion of intracellular GSH in peripheral lymphocytes. Effects of BSO administration on tumor cell GSH levels are being investigated. In addition, preclinical toxicology studies are under way for a combination of BSO and carboplatin.

Radiation and Chemotherapy Sensitizers

A randomized trial in patients with head and neck cancer is nearing completion and will establish the efficacy of SR 2508 and radiotherapy versus radiotherapy alone. Several pilot studies are ongoing. Phase I studies have defined the maximum-tolerated dose of SR 2508 given with brachytherapy based on preclinical data that suggest that SR 2508 may be more effective when given with low-dose rate radiotherapy. This approach is particularly promising in prostate cancer and brain tumors, and Phase II trials are being conducted in these disease sites. Phase I trials have been completed with the combination of SR 2508 and cyclophosphamide and one study is beginning to better define the optimum delivery schedule and mechanism of chemosensitization by this agent. The ECOG is planning Phase II trials in both small cell lung cancer and breast cancer. Several innovative pilot trials are under way that add SR 2508 to platinum-containing regimens based on preclinical data that the drug chemosensitizes the tumor to both cisplatin and carboplatin. Trials are now being designed that test the application of these combinations to ovarian cancer and autologous bone marrow transplantation.

Photodynamic therapy is a new modality for the treatment of cancer in which a light-activated drug is administered to a patient and becomes cytotoxic only upon exposure to light, which in most clinical applications is supplied by a red laser. Photofrin II is the most clinically advanced of these compounds and has demonstrated activity against a wide range of surface and intraluminal malignancies. A Phase I study at the NIH Clinical Center in intraabdominal malignancies is leading towards a GOG trial of photodynamic therapy for postoperative residual disease in ovarian cancer, and the SWOG is planning a bladder cancer trial. A major limitation of this approach is the necessity to deliver light to the tumor.

The Investigational Drug Branch of CTEP is working with the Radiation Research Program and the Developmental Therapeutics Program to help develop newer photo-therapeutic drugs with physicochemical properties that offer the possibility of broader applications of this technique to cancer treatment.

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In summary, the broad scope of research activities within the Division of Cancer Treatment encompasses both basic and clinical research efforts. Divisional responsibilities extend from the earliest identification of antitumor activity of a compound to the final phases of clinical testing in which the therapeutic activity of the agent or modality is clearly defined and the product is ready to be considered for licensing and approval by the FDA.

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Definitions of Contract Groupings

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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 that 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 BRMs.

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

Other - Contracts that 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 that 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	$46,535
	A. Compound Discovery - Subtotal	29,626
	1. Acquisition	11,025
	a. Natural Products	6,282
	b. Synthesis	4,743
	2. Screening	18,601
	a. In Vivo	6,037
	b. In Vitro	5,689
	c. Screening Support	6,875
	B. Compound Development	7,541
	1. Cancer	3,965
	2. AIDS	3,576
	C. Clinical Support	9,368
II.	Biologics Development	2,526
III.	Diagnostic Imaging	179
IV.	Radiation Development	3,045
V.	Clinical Trials - Subtotal	11,483
	A. Drug Evaluation (Phase I/II/III)	8,038
	B. Biological Evaluation (Phase I/II)	2,742
	C. Radiotherapy	653
	D. Other	50
VI.	Support to Intramural	2,768
VII.	Drug Purchases	3,000
	A. Cancer Drugs	2,000
	B. AIDS Drugs	1,000
VIII.	Program Mangement	700
	TOTAL DCT CONTRACTS	$70,236

TABLE II

DESCRIPTION OF CONTRACTS

IN THE

DIVISION OF CANCER TREATMENT

OFFICE OF THE DIRECTOR

JAPANESE FOUNDATION FOR CANCER RESEARCH (NO1 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. This contract was renewed in May, 1990, for three years.

TECHNICAL RESOURCE, INC. (NO1-CM5-7658)

This contract provides technical support services to the Office of the Director, DCT, 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 recompeted and the new award is expected to be made in July, 1990.

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 dependent 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-γ (IFN-γ); (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. This contract was due to expire in September of 1988; however, due to a shortage of 14G2a antibody, it was late in getting started. Antibody has now been provided for this contract by Brunswick Corporation. This contract expired on September 30, 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 Program 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 dependent 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. An approved protocol has been written for the study of 14.18 antibody, a chimeric version of the murine anti-GD2 antibody 14.G2a under study at the same institution under a previous BRMP contract (N01-CM4-7679). Preclinical studies are being performed to establish the monitoring assays for the trial, as well as to evaluate certain aspects of the clinical-grade antibody being produced. Large-scale production for the trial is currently underway. This is a cost reimbursement contract which was awarded March 31, 1989 and is scheduled to 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. Patient accrual for this trial has been closed after the treatment of 13 patients. Final analysis of immunological and clinical data is underway.

Another segment of this contract is a 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. The trial was delayed pending availability of yeast-derived GM-CSF from the company. Protocol accrual is now underway. This contract is scheduled to expire on December 31, 1990.

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 is scheduled to expire on September 29, 1993.

CLEVELAND CLINIC FOUNDATION (NO1-CM4-7673)

This Master Agreement supports a Phase Ib clinical trial of a murine monoclonal antibody (R24) which reacts to a surface antigen (GD3) present on many melanoma cells. Antitumor responses have been recorded in previous studies after administration of the antibody alone. In the current study, the antibody is given in combination with a chemotherapy regimen which has also been shown to have antitumor responses in melanoma: cisplatin and WR-2721. The trial is designed to determine toxicity of the combination in patients with metastatic malignant melanoma. Other endpoints of the study include: (1) investigation of effects of different antibody dose levels on in vivo binding of the antibody to melanoma cells; and (2) determination of mechanisms of potential synergy between R24 and the cisplatin/WR-2721 regimen. The results of this trial will be used to plan further studies with the same antibody or a chimeric version currently in preparation, in combination with chemotherapy and/or immunotherapy regimens. The present trial is also anticipated to influence the design of other protocols involving combinations of several such anti-melanoma antibodies. Commencement of the trial was delayed pending solution of production problems for the antibody R24. These problems have been solved. The trial is currently open and accruing patients.

Another segment of this contract is a Phase Ib study 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 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 (LAK) cells 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; and (4) to record objective tumor responses in this regimen. The clinical trial has been completed after the treatment of 18 evaluable patients. A maximal tolerated dose of IL-2 in this regimen was reached at 3.0×10^6 u/m^2/24 hours, with dose limiting toxicity being pulmonary at 4.5×10^6 u/m^2/24 hours. A preliminary analysis of data has been submitted and a final analysis is being prepared.

A Phase Ib study of macrophage activation by granulocyte-monocyte colony stimulating factor (GM-CSF) in patients with lung cancer is also being conducted. 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 pharmacokinetics, induction of anti-GM-CSF antibody, and changes in cytotoxic T-lymphocytes and natural killer activity. This study has been closed to patient accrual after 17 evaluable patients were treated. The dose-limiting toxicity was (reversible) pulmonary toxicity in 3/4 patients at the fourth dose level (500 ug/m^2/d 1-14 as with C.I.). The maximal tolerated dose was identified as 250 ug/m^2/d IV d 1-14. No clinical responses were seen. A preliminary analysis of the data has been submitted and a final analysis is underway. This contract is scheduled to expire November 30, 1990.

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. A protocol has been approved for a trial of recombinant human granulocyte-macrophage colony stimulating factor in patients with lung cancer. The trial is open for patient accrual. A second protocol has been submitted and is undergoing revision for the performance of a Phase Ia/b trial of interleukin-2 in combination with interleukin-4. This contract is scheduled to expire on June 29, 1994.

DAMON BIOTECH, INC. (NO1-CM6-7873)

The purpose of this contract, which is now in its third and final year, 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, at least 10 grams of the antibody by in vitro culture techniques. Following production of the bulk monoclonal antibody, the Contractor will perform the necessary purification testing and vialing as directed by the Project Officer in compliance 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 the 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 Contractor has already produced and delivered a total of 194 grams of 11 different antibodies, many of which are already in clincal trials. Two chimeric antibodies are scheduled for delivery late in calendar year 1990. This contract expired on August 31, 1990.

DAMON BIOTECH, INC. (N01-CM8-7253)

The purpose of this Task Order is to create a cell clone by molecular genetic techniques which produces human/mouse chimeric monoclonal antibodies to the epidermal growth factor receptor expressed in high concentrations on certain malignant cells. The starting point for the creation of the clone is the mouse hybridoma cell line (225) which makes mouse antibodies to the epidermal growth factor receptor. The Contractor must construct the cell line and then test its product for binding and biological activity. Although this Master Agreement Task Order is not required to produce large-scale quantities of the engineered antibody, clones must be selected which secrete antibody product in sufficiently high concentrations to make scale-up for clinical trials feasible.

Another segment of this Task Order is to create a cell clone by molecular genetic techniques which produces human/mouse chimeric monoclonal antibodies to the disiagloganglioside GD3 expressed on the cell surface of melanoma, neuroblastoma, and a subset of T-cells. The starting point for the creation of the clone is the mouse hybridoma cell line (R24) which makes mouse antibodies to the GD3 antigen. The Contractor must construct the cell line and then test its product for binding and biological activity. Although this Master Agreement Task Order is not required to produce large-scale quantities of the engineered antibody, clones must be selected which secrete antibody product in sufficiently high concentrations to make scale-up for clinical trials feasible.

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 is scheduled to expire September 9, 1992.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NO1-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 contract is currently supporting immune monitoring of several studies: (1) interleukin-1 beta in combination with 5FU; (2) interleukin-3 in patients receiving chemotherapy; (3) a Phase I study of macrophage-colony stimulating factor; (4) an open label study of patients who have shown benefit from therapy with IL-1β in a previous study; and (5) a Phase I study of interleukin-4. This is a cost reimbursement contract and is scheduled to expire on September 29, 1993.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NO1-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 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 Program of the Division of Cancer Treatment. The Contractor has established laboratory assays and performed preclinical evaluation of clinical-grade R24 monoclonal antibody, necessary to solve problems in production of that antibody for NCI trials. Protocols have been written for the study of R24 in GD3 positive lymphomas, as well as studies of an anti-EGF receptor monoclonal antibody in lung cancer. These protocols are undergoing final revision and are anticipated to start in mid-calendar year 1990. This a cost reimbursement contract which is scheduled to expire on March 14, 1994.

MEMORIAL SLOAN-KETTERING CANCER CENTER (NO1-CM4-7665)

This Phase Ib clinical trial is 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 fragment production. There was a delay in production of R24 which has been solved with the assistance of the Contractor, and production of the R24 fragments is being accomplished. This contract expired on June 30, 1990.

MOUNT SINAI SCHOOL OF MEDICINE (N01-CM6-7891)

This Master Agreement supports a Phase Ib clinical trial of a murine monoclonal antibody (LYM-1) which reacts to an antigen present on many non-Hodgkin's lymphoma cells. The antibody will be given in conjunction with interleukin-2 (IL-2) to investigate possible mechanisms of antitumor action of the combination, such as potentiation of antibody-dependent cellular cytotoxicity (ADCC) mediated by the antibody. The trial will also determine the human anti-mouse antibody response (HAMA) to the monoclonal antibody given in combination with IL-2. Clinical antitumor responses of the regimen will be recorded. The results of this trial will influence the design of later trials involving the combination of immunotherapy regimens with anti-lymphoma monoclonal antibodies. This trial is currently open for patient accrual. This contract expired on September 30, 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 has been produced for this contract by the Techniclone Corporation. The protocol is currently open for patient accrual. This contract is scheduled to expire on March 15, 1991.

SYNTHECELL CORPORATION (NCI-CM9-7579)

This Master Agreement contract is designed to synthesize various antisense oligodeoxynucleotides in amounts ranging from one to several hundred milligrams. The rationale for making these reagents is that they have been shown to block the action of certain oncogenes, growth factors, or cytokines which may be produced in excess amounts in malignant cells. Since various derivatives of these oligonucleotides have been reported in the literature, this contract will require the synthesis of specific unmodified and modified (phosphorothioates, alkylphosphotriesters, methylphosphonates, etc.) sequences of various lengths. It is anticipated that specific task orders under this Master Agreement will be awarded this fiscal year for: (1) the production of 100 mg quantities of eight (8) unmodified oligonucleotides (15-mers) in affecting the oncogenes, c-Myc and N-myc with respect to cell growth and differentiation; and (2) the synthesis of one mg amounts of four (4) phosphorothioates (20-27 mers) which affect natural killer cell receptor and cell functions. This contract was awarded in May, 1990.

TEXAS, UNIVERSITY OF (M.D. ANDERSON CANCER CENTER) (N01-CM6-7889)

This Master Agreement Order supports the performance of a Phase Ib clinical trial of a murine monoclonal antibody (14.G2a) to a surface antigen (GD2) found on a large fraction of human melanoma cells. The clinical protocol is designed to determine the toxicity and maximum tolerated dose of the antibody administered by a 5-day continuous intravenous infusion. Other important goals of the protocol are: (1) to study pharmacokinetics and biodistribution of the antibody; (2) in patients where excisional biopsies can be performed, to quantify the tumor uptake and binding of antibody and to determine effector cell phenotype; (3) to measure selected cellular and humoral immunologic parameters in peripheral blood; (4) to measure human anti-mouse antibody responses and determine the immunological response to dose escalation; and (5) to record antitumor responses to the treatment. The murine monoclonal antibody used for this trial has been produced at the Brunswick Corporation and patient accrual is underway. The results of this study will be used to plan further trials to evaluate combinations of anti-melanoma antibodies and cytokines, and to plan trials of mouse-human chimeric versions of such antibodies which are being developed. This contract is scheduled to expire on January 29, 1991.

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 IFN-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. 14.G2a is in production for this study. This contract is scheduled to expire on January 29, 1991.

TEXAS, UNIVERSITY OF (M.D. ANDERSON CANCER CENTER) (N01-CM9-7610)

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-dependent cellular cytotoxicity, tumor infiltrating lymphocytes 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 Program. This cost reimbursement contract and is scheduled to expire on May 31, 1994.

WISCONSIN, UNIVERSITY OF (NO1-CM4-7669)

This Master Agreement Order supports a Phase Ib clinical study of interleukin-2 (IL-2) based adoptive immunotherapy. The current trial design is partially based on the results of previous Phase Ib studies of IL-2 regimens, performed at the same institution, in which IL-2 was used to develop an endogenous population of effector cells, using a schedule with reduced toxicity. Later studies have used infusions of exogenously prepared effector cells, in this IL-2 therapeutic schedule. In the present trial design, patients with renal cell cancer will be studied. The objectives of the study are to determine toxicity, large-scale feasibility and immunologic antitumor effects of two repetitive 4-day cycles of IL-2 given as a continuous infusion followed by autologous lymphokine activated killer (LAK) cells and 10 days of t.i.d. bolus IL-2 at tolerable doses. Other objectives of the trial design include: (1) determination of the biological effects of this regimen with autologous LAK cells, in comparison with previous data using the four repetitive 4-day cycles of IL-2; (2) to determine whether IL-2 and autologous LAK cells given in this fashion can be administered safely in a non-ICU setting; (3) to measure in vitro effects of anti-CD3 monoclonal antibody, as a BRM, which will be used to enhance the generation of LAK cells; and (4) to record clinical antitumor effects of the regimen. The results of this study will be used to design further low-toxicity trials with IL-2 based adoptive immunotherapy, which may incorporate the anti-CD3 and/or other monoclonal antibodies.

Another segment of this contract supports a Phase Ib trial to assess the modulation by interferon-gamma (IFN-γ) of the Tag-72 antigen, reactive with monoclonal antibody B72.3, on ovarian cancer cells in patients with malignant ascites. Cohorts of patients have been treated with escalating doses of IFN-γ (Biogen) ranging from 0.1 MU to 50 MU IP weekly over a 5-week period, followed by monoclonal antibody first alone and then monoclonal antibody together with the OBD of IFN-γ. 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-γ antigen modulation trial is underway and results are still preliminary. The data show a positive effect of IFN on antigen density and shedding. A second clinical trial to evaluate murine monoclonal antibody + IFN-γ given systemically to patients with solid tumor, has been designed based on this experience and is under review. This contract is scheduled to expire on January 30, 1991.

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. Two protocols are currently being supported by this Contract, and are accruing patients: (1) a trial of interferon- and interleukin-2 (IL-2) in patients with advanced cancer incorporating prior BRMP-supported experience with these two agents at this institution; (2) a trial of murine anti-CD3 monoclonal antibody + IL-2 as immune activation in patients with cancer. This trial is designed to investigate the possible usefulness of this antibody as a biological response modifier to improve generation of effector cells. Both

trials are too early to report conclusive results. A third protocol is under review: augmentation of interferon-induced proteins in adenocarcinomas after exogenous interferon therapy. This protocol is designed to extend results developed under a previous BRMP contract (N)1-CM4-7669), in which antigens were unregulated on tumor cells by interferon; these antigens may be useful as targets for monoclonal antibody therapy, as studied in the intramural NIH laboratory of Dr. Jeffrey Schlom. This is a cost reimbursement contract which is scheduled to expire on September 29, 1993.

CANCER THERAPY EVALUATION PROGRAM

CALIFORNIA, UNIVERSITY OF (NO1-CM7-3702)

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

CHICAGO, UNIVERSITY OF (NO1-CM0-7301)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

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

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

EMMES CORPORATION (N01-CM6-7908)

This contract provides support to CTEP in two areas. The Contractor provides direct organizational data management and statistical support for specific clinical trials (currently the extramural LAK/IL-2 trials and Group C protocols for deoxycoformycin in hairy cell leukemia, fludarabine in CLL, and VM-26 in ALL). 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. The Contractor also provides information management assistance to the Clinical Investigations Branch professional staff in the analysis of methodology and data emanating from the extramural program.

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. This contract will expire on February 17, 1991 but the project is currently being recompted for award in Fiscal Year 1991.

JOHNS HOPKINS UNIVERSITY (NO1-CM0-7302)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

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. During the contract year, the

Contractor studied HMBA in Myelodysplastic Syndromes, Trimetrexate, Ipomeanol (including pharmacokinetic studies), Hepsulfam (including pharmacokinetic studies), Taxol/Cisplatin and TNF/VP-16. This contract expired on April 30, 1990.

LOYOLA UNIVERSITY (NO1-CM7-3704)

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

MARYLAND, UNIVERSITY OF (NO1-CM0-7303)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

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. During this contract year, the Contractor studied HMBA, Chloroquinoxaline Sulfonamide and Liposomal Doxorubicin. Pharmacokinetic studies have been undertaken as part of these trials. This contract expired on April 30, 1990.

MAYO FOUNDATION (NO1-CM0-7304)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

MAYO FOUNDATION (N01-CM0-7309)

This contract is one of three contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of early and high priority Phase II trials. The objectives of the contract are: a) when testing new agents which have just completed Phase I trials, to confirm that the dose and schedule chosen can be safely given in subsequent Phase II trials; b) to determine the antitumor activity of existing antitumor agents which can be administered in significantly higher doses when used with colony stimulating factors or other factors which modulate toxicity or antitumor activity; c) to determine the antitumor activity of combinations of antitumor agents and modalities; d) to evaluate laboratory parameters which may correlate with or predict for response; and e) to determine the spectrum of antitumor activity for new agents in selected human cancers. This contract was awarded on May 1, 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. During the contract year, the Contractor performed a Phase I study with Alpha Interferon, doxorubicin, and difluoromethylormithine and pilots of CTX, CBDCA, GM-CSF in advanced cancer and 5-FU + IFN-rA for advanced cancer. The Contractor studied the following agents in Phase II trials: Tumor Necrosis Factor (melanoma & lung); Ifosfamide/VP-16 (sarcoma); DHAC; 6-thioquanine in relapsed lymphoma; IV Nafidimide in advanced colorectal cancer; IV HXM in advanced ovarian cancer; and TNF for prostate cancer. This contract expired on April 30, 1990 and was replaced with contract N01-CM0-7309 for Phase II/III studies and N01-CM-07304 for Phase I studies.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (N01-CM0-7311)

This contract is one of three contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of early and high priority Phase II trials. The objectives of the contract are: a) when testing new agents which have just completed Phase I trials, to confirm that the dose and schedule chosen can be safely given in subsequent Phase II trials; b) to determine the antitumor activity of existing antitumor agents which can be administered in significantly higher doses when used with colony stimulating factors or other factors which modulate toxicity or antitumor activity; c) to determine the antitumor activity of combinations of antitumor agents and modalities; d) to evaluate laboratory parameters which may correlate with or predict for response; and e) to determine the spectrum of antitumor activity for new agents in selected human cancers. This contract was awarded on May 1, 1990.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (N01-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. During this contract year the

Contractor studied Hexamethylene bisacetamide; Deoxyspergualin (including pharmacokinetic studies); Chloroquinoxaline sulfonamide (including pharmacokinetic studies); and Liposomal encapsulated doxorubicin. The Contractor studied the following agents in Phase II trials: CHIP; Gallium; 5FU vs. 5FU & CPPD; CBDCA & VBL; Menogaril; ;CBDCA/VP vs. CPDD/VP; Trimetrexate; Gallium vs. Etidronate; High dose MTX; HMBA; Didenmin; High-dose MVAC; R24 High vs. Low; IFOS/MESNA; Doxorubincin; and Suramin. This contract expired on April 30, 1990.

MONTEFIORE MEDICAL CENTER (NO1-CM7-3705)

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

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

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

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. During the contract year the Contractor studied Fazarabine, Deoxycoformycin and Pludarabine Phosphate/Deoxycoformycin. Pharmacokinetic and immunolgic studies have been accomplished on most of these patients. This contract expired on April 30, 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 Contractor developed a reversed-phase, isocratic HPLC assay to quantitate Suramin in patient plasma samples. Suramin demonstrated activity in prostatic cancer patients and the development of this agent has become a high

priority for DCT. The assay enhances the efficacy of the extraction of Suramin from plasma, reduces the sample manipulations and time required for the assay and uses readily available equipment. The Contractor validated the assay at new centers conducting clinical trials with Suramin. The Contractor will analyze samples from centers lacking liquid chromatography expertise. The contractor performed the pharmacokinetic analysis of plasma samples from patients treated in the BRMP protocol utilizing FAA and IL-2. Studies are also continuing with Amonafide and the phenotyping of patients with respect to their N-acetylation ability.

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 tracking.

TEXAS, UNIVERSITY OF, HEALTH SCIENCE CENTER (NO1-CM0-7305)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

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. During the contract year, the Contractor conducted Phase I studies with Deoxyspergualin 120 hour continuous infusion, Hepsulfam (including pharmacokinetic studies), Tumor necrosis factor (including pharmacokinetic studies), Lipsomal Doxorubicin (including pharmacokinetic studies). This contract expired on April 30, 1990.

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

This Contractor is part of the NCI IL/2-IAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) results in malignant melanoma and colorectal carcinoma. The response rate in ILWG studies in renal carcinoma was half that observed in the SB and was probably related to the greater tumor burden in patients treated by the ILWG. These results were used to place IL-2/LAK into the Modified Group C Program. In subsequent studies, the ILWG was not able to improve upon these results by modifying the IL-2/LAK regimen. Currently the ILWG is evaluating IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma have also been initiated.

TEXAS, UNIVERSITY OF, SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (NO1-CM0-7310)

This contract is one of three contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of early and high priority Phase II trials. The objectives of the contract are: a) when testing new agents which have just completed Phase I trials, to confirm that the dose and schedule chosen can be safely given in subsequent Phase II trials; b) to determine the antitumor activity of existing antitumor agents which can be administered in significantly higher doses when used with colony stimulating factors or other factors which modulate toxicity or antitumor activity; c) to determine the antitumor activity of combinations of antitumor agents and modalities; d) to evaluate laboratory parameters which may correlate with or predict for response; and e) to determine the spectrum of antitumor activity for new agents in selected human cancers. This contract was awarded on May 1, 1990.

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. During the contract year, the Contractor studied the following agents in Phase I trials: Bensisoquinolinedione; Tricyclic Nucleotide Phosphate, Triciribine; Human Anti-colorectal carcinoma monoclonal antibody; Interleukin 2; Fazarabine; and Xomazyme.

During this contract year the Contractor studied the following agents in Phase II trials: Taxol; Carboplatin; Dihydro-5-Azacytidine; Trimetrexate; Methyl CCNU in combination with 5-FU plus folinic acid; Didemnin B; Fludarabine plus prednisone; high dose melphalan and total body irradiation with autologous bone marrow rescue; Carboplatin plus cisplatin; IV Melphalan; Carboplatin and bleomycin; Menogaril; IV 6-Thioguanine; 5-FU and high-dose Folinic Acid; Tiazofurin; Amonafide; TPDC-FUHU; Homoharringtonine; Fludarabine; Gallium-Nitrate; Interferon alpha and 5-FU; Suramin, Arabinosyl-5-Azacytidine; arabinsylcytosine; Interleukin-2; Tumor Necrosis Factor and Interleukin-2; Continuous infusion Carboplatin and 5-FU followed by procarbazine; and Cyclophosphamide, Interleukin-2 and Tumor Infiltrating Lymphocytes. This contract expired on April 30, 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 contract investigators and certain other categories of clinical research using NCI-sponsored investigational drugs. This service has four components--(1) a central data management resource for investigators conducting Phase I clinical 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) attending a sample of cooperative group on-site audits as observers; and (4) on-site auditing of all other individual investigators conducting investigational trials.

WARNER-LAMBERT COMPANY (NO1-CM3-7285)

This is a no-cost contract which provides for the development and marketing of diaziquone (AZQ) as an antitumor agent. Its purpose is to facilitate development of the agent through Phase III, prepare the New Drug Application (NDA) for submission to the Food and Drug Administration (FDA) and market the agent following FDA approval of the NDA. Warner-Lambert is collecting the data from their Phase III study of diaziquone and BCNU in primary brain tumors in addition to the data from a study at Duke which compared diaziquone with BCNU in an adjuvant setting. Warner-Lambert will use the data from their study and the Duke study for their NDA submission to FDA. This contract will be phased out during this fiscal year.

WISCONSIN, UNIVERSITY OF (NO1-CM0-7306)

This contract is one of six contracts awarded this fiscal year as part of a recompetition to provide a resource for the conduct of Phase I clinical trials of anticancer agents. The objectives of the contract are: a) to define the acute toxicities of new anticancer agents in patients with advanced cancer; b) to redefine the acute toxicities and pharmacokinetics of anticancer agents administered in combinations with agents to modulate toxicity or antitumor effect; c) to provide information on the pharmacokinetic characteristics (absorption, distribution, metabolism, and elimination) and pharmacodynamics of selected antitumor agents; and d) to determine a treatment regimen suitable for evaluation of antitumor activity in Phase II trials. This contract was awarded on May 1, 1990.

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. During the contract year the Contractor studied Fazarabine (including pharmacokinetics); Whole Body Hyperthermia and Carboplatin (including pharmacokinetics); 5-FU, Dipyridamole and Leucovorin (including pharmacokinetics); SR2508 and Cyclophosphamide; Tumor Necrosis Factor; Tumor Necrosis Factor and Gamma Interferon. Pharmacokinetic studies were performed on selected patients in all Phase I studies. This contract expired on April 30, 1990.

CLINICAL ONCOLOGY PROGRAM

ADVANCED BIOSCIENCES, INC. (NO1-CM8-7236)

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

DEPARTMENT OF ENERGY (ARGONNE NATIONAL LABORATORIES) (YO1-CM9-0148)

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 (YO1-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.

LABORATORY ANIMAL SERVICES, INC. (NO1-CM9-7596)

The Hazelton Laboratories, Inc. animal support contract for the Surgery Branch expired on August 31, 1989. The contract was recompeted and awarded for a period of five years from September 1, 1989 to August 31, 1994 to Laboratory Animal Services, Inc. This contract supports the research efforts of the Surgery Branch through the provision, maintenance and transfer of tumor bearing laboratory animal models for investigation. The contractor also provides Surgery Branch investigators with 24-hour access to animals and workspace for the performance of experimental procedures.

LIBRARY OF CONGRESS (YO1-CM5-0133)

The Library of Congress will provide administrative services to the Medicine Branch to facilitate procurement, installation and training of library support and information retrieval services and associated technical services under the FEDLINK bylaws. This agreement will be phased out in fiscal year 1990.

MEDICAL ILLNESS COUNSELING CENTER (NO1-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 (YO1-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. This contract is undergoing recompetition in Fiscal Year 1990.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (YO1-CM9-0166)

The USUHS will provide faculty and medical staff positions to support the collaborative efforts for research and educational opportunities between the USUHS, National Cancer Institute, 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-CM9-0162)

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-CM9-0167)

The purpose of this Memorandum of Understanding (MOU) is to permit collaborative efforts among the USUHS, National Cancer Institute (NCI), and 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 HEATLH 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. This agreement will be phased out during Fiscal Year 1990.

AEROJET STRATEGIC PROPULSION COMPANY (N01-CM8-7273)

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 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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

ALABAMA, UNIVERSITY OF (N01-CM0-7335)

This is one of the 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.

ALABAMA, UNIVERSITY OF (N01-CM8-7267)

This contract is for the design and chemical synthesis of a variety of compounds for evaluation as potential anti-AIDS agents. The types of compounds include: (1) congeners of confirmed screening leads to enhance activity or potency; (2) prodrugs of active leads, with structural modifications that may provide altered pharmacokinetics, altered drug transport, improved bioavailability through increased water solubility, or increased chemical stability; and (3) other altered structures that possess elements of both congener and prodrug of the active lead. Modifications of a lead may also include partial structures. Structural types include synthetics and compounds related to natural products, such as alkaloids, nucleosides, peptides, and various heterocycles. Active leads are generally organic compounds but may also include metal complexes.

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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

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. This contract is being recompeted.

APPLIED ANALYTICAL INDUSTRIES (N01-CM9-7571)

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.

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

This contract provides a resource for preclinical 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 intravenous, subcutaneous (bolus and/or infusion), and oral dosing in mice, rats, and dogs; and (4) identification and pharmacokinetic analysis of drug metabolites. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Data obtained in these investigations are used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Preclinical pharmacology studies of potential anti-AIDS compounds 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-CM6-7927)

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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

ASH STEVENS, INC. (N01-CM9-7592)

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 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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

ATLANTIC RESEARCH CORPORATION (N01-CM0-7313)

This contract agreement includes work on requirements analysis, program design and coding, debugging, user instruction, and installation of fully tested code as well as development of user and system documentation. It also maintains and services two laser printers and a microVAX in Executive Plaza North. This contract was awarded in March 1990 for a 5-year period.

AUSTRALIAN INSTITUTE OF MARINE SCIENCE (N01-CM0-7322)

This contract furnishes approximately 1,000 shallow-water marine organisms per year for antitumor and anti-AIDS evaluations. The marine organisms are selected to represent the greatest possible taxonomic and environmental diversity within a large number of Indo-Pacific collecting sites. The contract also allows for bulk recollections of organisms of interest for further study. This 2-year contract was initiated in November 1989, and the first delivery of organisms is expected in June 1990.

BATELLE MEMORIAL INSTITUTE (N01-CM8-7233)

This Master Agreement for Master Agreement Orders in the large-scale recollection of shallow-water marine organisms for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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.

BATELLE MEMORIAL INSTITUTE (N01-CM8-7280)

This Master Agreement for Master Agreement Orders in the large-scale recollection of deep-water marine organisms for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. This Master Agreement Order mechanism enables the prompt solicitation in this area from a pool of qualified contractors. As yet, no project has been assigned to the contractor.

BATTELLE MEMORIAL INSTITUTE (N01-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 sutides 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 second year of a 3-year contract.

BEN VENUE LABORATORIES, INC. (N01-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 Practices regulations. Other responsibilities include quality control testing, packaging, labeling, and distribution of the final product to the National Cancer Institute. This contract is being recompeted.

BEN VENUE LABORATORIES, INC. (N01-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.

CAPITAL TECHNOLOGY INFORMATION SYSTEMS (N01-CM9-7600)

This contract provides for the installation and maintenance of the DTP AIDS Screening Database. Installation included the complete design, coding, and implementation of the AIDS Screening Database in ORACLE. Ongoing activities include monitoring system performance, trouble-shooting, debugging, documentation, and enhancements to the overall system. This contract was awarded in February 1989 for a 5-year period.

CHARLES RIVER LABORATORIES (N01-CM9-7575)

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

CHICAGO, UNIVERSITY OF (N01-CM9-7567)

This contract is for the acquisition of fresh surgical specimens of human breast cancer and the development of cell lines suitable for use in the DTP screening program. An attempt is made to establish surgical material in both cell-culture media and in athymic mice. A complete clinical history, as well as culturing detail and other characterization information, is provided for each line.

ERC BIOSERVICES (N01-CM0-7320) (Formerly N01-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, INC. (N01-CM8-7276)

This contract is for the continuing maintenance and improvement of the Drug Information System (DIS), Natural Products Repository Support System (NPRSS), and Pharmaceutical Data System (PDS). These systems are large computerized systems designed to support the many aspects of DTP, in drug prescreens for AIDS and cancer and the clinical development of compounds for further testing. Specifically, the contractor is responsible for providing (1) the continued updating of the DIS, NPRSS, and PDS to reflect the most current information on all compounds contained in the systems; (2) detection and correction of any problems within the systems; and (3) upgrading the systems to the newest technology available.

FEIN-MARQUART ASSOCIATES, INC. (N01-CM9-7608)

This contract is one of several contracts that provide computer software design, programming, implementation, and maintenance support to DTP. During the period of this report, the primary thrust of this contract has been in the area of the Natural Product Repository Support System (NPRSS). Initially, the needs of the Extraction and Grinding laboratory were reevaluated and that portion of the NPRSS reprogrammed. During the implementation phase, communications problems not related to the software or contractor were discovered and resolved. In the second half of the report period, the entire NPRSS was analyzed. The redesign and programming as an ORACLE database is well underway at this time. This contract was awarded in February 1989 for a 2-year period.

GENZYME (Formerly Integrated Genetics, Inc.) (N44-CM8-3717)
(SMALL BUSINESS INNOVATION RESEARCH Program)

This contractor is developing a panel of human solid tumor sublines that express the MDR (multidrug resistance) phenotype. The sublines are obtained by classic techniques involving selection of drug-resistant clones after repeated exposure of cells to drug-containing media or preferably by newer gene transfer techniques. New sublines are characterized for multidrug resistance, mdr gene copy number, growth characteristics, stability, etc. These sublines should be

valuable in disease-oriented screening projects in the search for new anticancer agents with activity against drug-resistant tumors and for studies of the MDR phenotype. This contract will expire this fiscal year.

GEORGIA, UNIVERSITY OF (N01-CM7-3712)

This contract 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 are developed are used to verify the stability of NCI's investigational drugs during the clinical trials and are supplied to the U.S. Food and Drug Administration in support of NCI's IND filings. This contractor also has the responsibility of conducting reserve sample inspections as required by the U.S. FDA Current Good Manufacturing Practices.

GEORGIA TECH RESEARCH CORPORATION (N01-CM8-7269)

This contract provides for the design and chemical synthesis of a variety of compounds for evaluation as potential anti-AIDS agents. The specific objectives of this project are: (1) to synthesize congeners of synthetic compounds with confirmed activity; (2) to design and synthesize prodrugs and other compounds that posess elements of both congener and prodrug; and (3) to synthesize compounds related to products of natural origin and other related heterocycles. These products may include partial structures of analogs and novel heterocycles.

HARBOR BRANCH OCEANOGRAPHIC INSTITUTE (N01-CM8-7292)

From September 1988 to November 1989, this contract furnished 1,162 shallow-water marine organisms for antitumor and anti-AIDS evaluations. The marine organisms were selected to represent the greatest possible taxonomic and environmental diversity within a large number of Indo-Pacific collecting sites. The contract was completed in November 1989.

H.G. PARS PHARMACEUTICAL LABS., INC. (N01-CM0-7339)

This is one of the Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds that have been identified by program for development. Unique compounds from the literature, with reported biological activity, that 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.

HARLAN/SPRAGUE-DAWLEY, INC. (N01-CM2-3911)

This contract operates the Animal Production Area at the Frederick Cancer Research and Development Center (FCRDC). 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 FCRDC 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 FCRDC. Animals are also sent from the FCRDC to other NCI-funded research activities.

HARLAN/SPRAGUE-DAWLEY, INC. (N01-CM9-7623)

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

HAUSER CHEMICAL RESEARCH, INC. (N01-CM8-7272)

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.

HAUSER CHEMICAL RESEARCH, INC. (N01-CM9-7554)

This Master Agreement for Master Agreement Orders in the large-scale isolation of antitumor agents from natural source 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. Assignments have been made for the extraction and isolation of taxol (NSC 125973) from the bark of Taxus brevifolia.

HAWAII, UNIVERSITY OF (AT MANOA) (N01-CM6-7745)

The goals of this contract are: (1) the cryogenic culture of 900 cyanobacteria over a 5-year period; (2) preparation of extracts from the above-mentioned cyanobacteria; (3) delivery of cryopreserved cultures to the NCI repository; and (4) to conduct large-scale regrowth of those isolates designated by the NCI. Currently about 300 strains of cyanobacteria are processed per year and a number of cyanobacterial extracts have shown in vitro antiviral activity. A number of isolates have been selected for reculture so that additional material can be obtained for further testing.

HAWAII, UNIVERSITY OF (AT MANOA) (N01-CM8-7282)

This Master Agreement for Master Agreement Orders in the large-scale recollection of plants for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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.

ILLINOIS, UNIVERSITY OF (N01-CM0-7345)

This Master Agreement for Master Agreement Orders in the large-scale recollection of plants for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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.

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 the NCI for extraction and testing in the anticancer and AIDS-antiviral screens. To date, over 6,000 samples have been collected from Indonesia, Malaysia, Nepal, Papua New Guinea, Philippines, and Thailand.

ILLINOIS, UNIVERSITY OF (N01-CM8-7226)

This contractor provides a bimonthly listing of all new natural products that have appeared in the world literature that are of potential interest as test candidates for the NCI HIV and/or antitumor screens. The listings include chemical structures, reported biological activities, amounts isolated, and bibliographic citations. Full reprints of the papers, as well as those of publications on natural product SAR, are provided. The contractor will, on request, perform retrospective searches of families and genera of interest to the screening program. This contract is in the second of a 5-year award period which ends June 29, 1993.

IOWA, UNIVERSITY OF (N01-CM9-7572)

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.

IOWA, UNIVERSITY OF (N01-CM9-7577)

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, this 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.

KANSAS, UNIVERSITY OF (N01-CM9-7576)

This contract investigates approaches to resolve difficult dosage form development problems not amenable to usual solubilization or stabilization methods. This 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.

LELAND B. STANFORD, JR., UNIVERSITY OF (N01-CM9-7568)

This contract is for the procurement of human prostatic cancer material and the development of cell lines suitable for use in the DTP screening program. Fresh primary prostate cancer tissue is acquired at the time of surgery, processed, and established as primary cell cultures. A complete clinical history, as well as culturing detail and other characterization information, is provided for each line.

MARTEK BIOSCIENCES CORPORATION (N01-CM8-7283)

This Master Agreement for Master Agreement Orders in the large-scale recultivation of phototrophic microorganisms was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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 recently been assigned the task of large-scale culturing of the cyanobacteria, Lyngbya lagerhemii and Phormidium tenue, as sources of active sulfolipids.

MARTEK BIOSCIENCES CORPORATION (N01-CM9-7615)

The overall objective of this contract is to culture 600 strains of protozoa and produce extracts, which will be tested for antitumor and anti-AIDS activity. While considerable numbers of fungi and bacteria have been tested for antitumor and antiviral activity, protozoans, a diverse group of organisms, have received little study. Over 200 protozoans are in culture, and extracts from over 60 strains have been provided to NCI for testing.

MAYO FOUNDATION (N01-CM9-7618)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data that will be used to improve both the interpretation of preclinical toxicology data, as well as to improve 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 that 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 that 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 that was recompeted and awarded on April 30, 1989.

MIAMI, UNIVERSITY OF (N01-CM6-7877)

This contract provides for a complete pathological, parasitical, and microbiological work-up of breeding stock primarily sent from barrier room animal 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 Officer.

MIDWEST RESEARCH INSTITUTE (N01-CM7-3713)

Midwest Research Institute is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations that 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.

MIDWEST RESEARCH INSTITUTE (N01-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 to an INDA. This is the third year of a 5-year contract.

MIDWEST RESEARCH INSTITUTE (N01-CM8-7228)

Midwest Research Institute is one of two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations that are to be investigated in the pharmacological, toxicological, and clinical phases 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.

MISSOURI, UNIVERSITY OF (N01-CM6-7723)

This contract provides for a complete pathological, parasitical, and microbiological work-up of breeding stock primarily sent from barrier room animal 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-CM0-7346)

This Master Agreement for Master Agreement Orders in the large-scale recollection of plants for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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.

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 testing in the anticancer and AIDS-antiviral screens. To date, over 6,000 samples have been collected from Cameroon, Central African Republic, Gabon, and Tanzania.

NAPROTECH INC. (N01-CM9-7562)

The function of this contract was to provide a bimonthly listing of all new natural products that appeared in the world literature that were of potential interest as test candidates for the NCI antitumor screens. The listings included chemical structures, reported biological activities, amounts isolated, and bibliographic citations. Full reprints of the papers, as well as those of publications on natural product SAR, were provided as well. The contractor would, on request, perform retrospective searches of families and genera of interest to the screening program. This 1-year contract with an option to renew for two additional years was found to significantly overlap N01-CM8-7226. The option to renew was allowed to lapse on December 31, 1989.

NATIONAL ACADEMY OF SCIENCES (N01-CM0-7316)

This contract serves to develop (1) standards for animal care and maintenance, (2) shipping standards for the various species of laboratory animals, (3) standards for nomenclature used to identify stocks and strains of laboratory animals, (4) standards for animal maintenance in the research laboratory, and (5) laboratory animal procurement standards. These standards are formulated by

an ad hoc committee whose membership represents commercial animal production facilities, governmental and academic institutions, and nonprofit research institutions.

NEW MEXICO STATE UNIVERSITY (N01-CM8-7278)

This contract provides for chemical resynthesis 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, antisense nucleic acids, etc.

NEW YORK BOTANICAL GARDEN (N01-CM0-7347)

This Master Agreement for Master Agreement Orders in the large-scale recollection of plants for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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.

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 testing in the anticancer and AIDS-antiviral screens. To date, over 5,000 samples have been collected from 13 countries, including more than 300 medicinal plants used by traditional healers in Belize.

NORTH CAROLINA, UNIVERSITY OF (N01-CM9-7627)

This contract investigates approaches to resolve difficult dosage form development problems not amenable to usual solubilization or stabilization methods. This 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.

NORTHWESTERN UNIVERSITY (N01-CM8-7257)

This contract is designed to monitor and maintain genetic control of 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.

NORTHWESTERN UNIVERSITY (N01-CM9-7628)

This contract, entitled "Procurement of Prostate Cancer Cell Lines" acquires fresh surgical or autopsy prostate material for the development of cell lines to be used in the DTP screening program. Material is placed into media and/or

athymic mice, and an attempt is made to develop cell lines suitable for use in this screen. Clinical histories and other pertinent information is provided for each line.

OHIO STATE UNIVERSITY RESEARCH FOUNDATION (N01-CM9-7619)

Preclinical pharmacology studies of antitumor agents under development by DTP are conducted under this contract to collect data that will be used to improve both the interpretation of preclinical toxicology data and 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 that 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 that 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 will be alerted to their potential human significance.

PATHOLOGY ASSOCIATES, INC. (N01-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 provide: (1) pathology quality assurance review of completed studies and studies in progress; (2) pathology support in the form of site visits, photomicrography, slide preparation, performance of necropsies, and histopathologic diagnosis of lesions; (3) veterinary support such as site visits, the development of special surginal procedures, and instruction in these procedures; and (4) equipment storage, maintenance, and shipment. This is the third yeard of a 5-year contract.

PHARM-ECO LABORATORIES, INC. (N01-CM6-7928)

This service preparative contract provides for 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 commerical sources in the amounts and/or quality required. The major thrust of the effort of this contract is the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is being recompeted.

PHARM-ECO LABORATORIES, INC. (N01-CM9-7587)

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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This is the first year of a 3-year contract.

PHARM-ECO LABORATORIES, INC. (N01-CM9-7590)

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 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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

POLYSCIENCES, INC. (N01-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.

POLYSCIENCES, INC. (N01-CM9-7621)

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*.

PROGRAM RESOURCES, INC. (N01-C07-4102)

This contractor is located at the Frederick Cancer Research and Development Center (FCRDC) in Frederick, Maryland, and is divided into the following projects:

AIDS Chemical Prep Laboratory (Project 9042775102). This project is responsible for the appropriate weighing, solubilization, partial chemical characterization, and 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 9042774201). 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 (Project 9042762705). This project provides the research and development capacity, facilities, and staff to develop the necessary technology and implement the screening of large numbers of anti-HIV compounds annually in a safe, semiautomated 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 9042761301). 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 to be active *in vitro*.

AIDS Natural Products Laboratory (Project 9042764301). 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 9042775105). This project provides freezer storage space and cataloging support for natural product collections and for extracted natural products.

AIDS Protease Inhibition (Project 9042776101). 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 9041993413). 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 - Cancer (Project 9041775107). 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 a project is not clearly defined.

Biological Testing Branch (BTB) Supplies - Cancer (Project 9041415111). This project permits BTB personnel to utilize the PRI warehouse for obtaining office supplies.

Biological Testing Branch (BTB) Support - Cancer (Project 9041775103). This contract provides for two Information Specialists in the Office of the BTB.

Cell Line Development (Project 9041775109). This effort provides partial support for cell-line development from human tumors, particularly providing specialized characterization support.

Computer Building (Project 9041993416). This project provides for the erection of a modular building to house the DTP dedicated computer and for supportive contractor personnel.

Computer Support - Cancer (Project 9041774201). 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 - Cancer & AIDS (Project 9041993414). This project provides for the erection of modular office space for DTP personnel assigned to FCRDC.

DTP Chemical Prep Lab (Project 9041775102). This project is responsible for the appropriate weighing, solubilization, partial chemical characterization, and 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.

Fermentation Pilog Plant Renovation - Cancer & AIDS (Project 9041993415). This project provides for the renovation of Building 472, which will be utilized for expanded production of selected natural product materials.

Fungi Evaluation - AIDS (Project 9042765202). This project supports the growth of selected fungal microbes in order to obtain sufficient quantities for extraction and testing in the AIDS in vitro screen for natural products. "Actives" from the in vitro screen will be regrown for further in vitro and in vivo testing.

Fungi Evaluation - Cancer (Project 9041765202). This project supports the growth of selected fungal microbes in order to obtain sufficient quantities for extraction and testing in the Cancer in vitro screen for natural products. "Actives" from the in vitro screen will be regrown for further in vitro and in vivo testing.

Genetic Monitoring - Cancer (Project 9041762702). This project monitors all Biological Testing Branch contract rat colonies for genetic purity. In addition, this project also monitors the starts received from VRB-NIH, both pre- and post-rederivation.

Gut Flora Monitoring - Cancer (Project 9041762703). This segment receives animals weekly from DTP's Primary Genetic Centers. The animals originate from isolator colonies at the Genetic Centers, and the animals are monitored for gut flora and possible virus contamination. The animals are sent weekly on a schedule made up by the Biological Testing Branch.

Human Tumor Procurement and Preparation - Cancer (Project 9041775104). 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 - Cancer (Project 9041765201). This project provides in vitro cell-line development support. The DTP goal is to develop 60 to 100 well-characterized cell lines, with available patient histories as soon as possible.

In Vitro Cell Line Screen - Natural Products - Cancer (Project 9041775203). This 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 1990. Approximately 60 human tumor cell lines are included in each test.

In Vitro Cell Line Screen - Synthetic Compounds - Cancer (Project 9041775202). This project provices in vitro screening support, primarily for synthetic compounds. Capacity is expected to reach an annualized level of approximately 10,000 tests in FY 1990. Approximately 60 human tumor cell lines are included in each test.

In Vitro Cell Line Support - Cancer (Project 9041775201). This project purchases supplies and equipment that 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 - Cancer (Project 9041762701). Human tumor cell lines are developed for use as models for in vivo drug evaluations and for selected testing of in vitro "actives."

LAK Cell Media Evaluation - Cancer (Project 9041415113). This segment represents a modest shared service support effort for evaluating selected media for LAK cell growth.

Laboratory of Drug Discovery Research and Development (LDDRD) Support (Project 9041415111). This project provides service support, including materials, supplies, and equipment purchases for the LDDRD.

Marine Bacteria Lab - AIDS (Project 9042765203). This effort supports the culturing, extraction, and preservation of marine anaerobic organisms that are submitted to the AIDS in vitro screen. "Actives" from the screen will be recultured in sufficient amounts for further in vitro and in vivo testing.

Marine Bacteria Lab - Cancer (Project 9041765203). This effort supports the culturing, extraction, and preservation of marine anaerobic organisms that are submitted to the cancer in vitro screen. "Actives" from the screen will be recultured in sufficient amounts for further in vitro and in vivo testing.

Natural Products Extractions - Cancer (Project 9041764301). This project provides crude natural product extracts (plant and marine) in support of the in vitro and in vivo testing programs.

Natural Products Isolation - AIDS (Project 9042764304). This project assists with the structural identification of agents from "actives" identified by the AIDS in vitro screen. This project also supports efforts of visiting scientists who work in this area.

Natural Products Isolation - Cancer (Project 9041764304). This project assists with the structural identification of agents from "actives" identified by cancer in vitro/in vivo testing. This project also supports efforts of visiting scientists who work in this area.

Natural Products Repository - Cancer (Project 9041775105). This project provides freezer storage space and cataloging support for natural product collections and for extracted natural products.

Operational Support for Harlan/Sprague Dawley - Cancer (Project 9040202118). This project provides Work Orders and Shared Service type functions for Harlan/Sprague-Dawley Animal Production. It serves as a method for paying PRI for services performed at the Animal Production Area at Frederick.

Renovations for APA/HSP - Cancer (Project 9040993511). This project permits PRI to perform renovations for the Animal Production Area (APA) utilizing subcontractors.

Renovations for Building 313 - AIDS (Project 9042993419). Renovation of Building 313 will provide space to support expansion efforts for agents identified as active against AIDS.

Renovations for Building 325 - AIDS (Project 9041993418). Renovation of Building 325 will provide space to support the cultivation of selected fungal microbes for submission to the in vitro AIDS screen and will also support natural products isolation and identification efforts.

Renovations for Building 431 - Cancer & AIDS (Project 9041993411). 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.

Renovations for Building 432 - Cancer (Project 9041993412). This project provides support for renovating and equipping cancer in vitro screening laboratories. These laboratories are now completed and equipped.

Renovations of Five Animal Production Area (APA) Buildings - Cancer & AIDS (Project 9041993417). This project 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 - Cancer (Project 9041762704). The contractor 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 - Cancer (Project 9041775106). This project provides limited screening support for natural products isolation.

Taxol Isolation - Cancer (Project 9041764302). This is a shared service project which provides taxol isolation capabilities from crude raw materials.

Tumor Bank - Cancer (Project 9041775101). This project has as its major goal, the maintenance of approximately 40,000 frozen tumor vials. Needed tumors are furnished to the various DTP laboratories, as well as to other research institutions, both domestic and foreign. Tumors are supplied both in vivo and in vitro.

Visiting Scientist Support - AIDS (Project 9042775108). This effort supports travel and per diem expenses for short-term visits of scientists, particularly from Third World countries, who are participating in the collection of identified natural products samples for AIDS testing. The exchange of information provided through these visits has been of mutual benefit for DTP and for the countries of origination.

Visiting Scientist Support - Cancer (Project 9041775108). This effort supports travel and per diem expenses for short-term visits of scientists, particularly from Third World countries, who are participating in the collection of identified natural products samples for cancer testing. The exchange of information provided through these visits has been of mutual benefit for DTP and for the countries of origination.

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 (1) watersolubility; (2) hydrolytic stability; and (3) 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 to (1) synthesize congeners of synthetic compounds with confirmed activity; (2) design and synthesize prodrugs and other compounds that possess elements of both congener and prodrug; (3) synthesize compounds related to products of natural origin and other related heterocycles; and (4) synthesize antisense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

RESEARCH TRIANGLE INSTITUTE (N01-CM0-7330)

This is one of the Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds that 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.

RESEARCH TRIANGLE INSTITUTE (N01-CM7-3714)

Research Triangle Institute is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations that 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.

RESEARCH TRIANGLE INSTITUTE (N01-CM8-7227)

This contract provides for the synthesis of radiolabeled 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 radiolabeled 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.

RICERCA, INC. (N01-CM0-7331)

This is one of the Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds that 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)

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 that support the production colonies. Both the pedigreed expansion and production colonies are maintained in a barrier environment. Offspring from the production colonies are used for the many NCI research activities.

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 that will be used to improve both the interpretation of preclinical toxicology data and 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 that 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 will be alerted to their potential human significance.

SOUTHERN RESEARCH INSTITUTE (N01-CM0-7315)

This newly recompeted contract was awarded in February 1990, for a 5-year period. The objective of the project is to evaluate compounds for anticancer activity in experimental in vivo tumor models. Studies focus on agents identified by the Developmental Therapeutics Program's disease-oriented human tumor cell line screen and employ human tumors growing in immune-deficient (athymic) mice. Some studies may involve murine tumors growing in pathogen-free immune-competent mice. A diversity of nonroutine in vivo experiments are designed and conducted to optimize antitumor activity and evaluate the drug's therapeutic potential. In addition, capacity on the contract is maintained to explore new chemotherapeutic approaches requiring in vivo evaluation, and to provide a resource for specialized in vivo expertise for the National Cooperative Drug Discovery Groups.

SOUTHERN RESEARCH INSTITUTE (N01-CM0-7329)

This is one of the 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.

SOUTHERN RESEARCH INSTITUTE (N01-CM7-3726)

The major objective of the 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 terminated in February 1990.

SOUTHERN RESEARCH INSTITUTE (N01-CM8-7229)

This contract is to provide for the resynthesis of a variety of organic and/or inorganic compounds of interest for the AIDS antiviral or antitumor screens. These compounds consist of candidates of interest selected from the literature and/or lead compounds that are no longer available from the original sources. The majority of assignments involve the preparation of structures for which detailed experimental procedures are available. The contract is in the second year of a 3-year award period and will be recompeted.

SOUTHERN RESEARCH INSTITUTE (N01-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; anti-HIV activity of serum from animals treated with potential AIDS drugs; and intracellular drug pharmacology and biochemical studies. This is the third year of a 3-year contract.

SOUTHERN RESEARCH INSTITUTE (N01-CM8-7237)

This contract "Primary Screening of Compounds Against HTLV-III/LAV (Human AIDS Virus)" provides a capacity of 20,000 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 (N01-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 that 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.

SPRINGBORN RESEARCH INSTITUTE (N01-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 to an INDA. This is the third year of a 5-year contract.

SOUTHERN RESEARCH INSTITUTE (N01-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 to an INDA. This is the third year of a 5-year contract.

SOUTHERN RESEARCH INSTITUTE (N01-CM8-7274)

The objectives of this project are to explore the influence of dose, exposure time, and route of administration on the in vivo antiviral activity of new compounds that are known to inhibit the growth and/or cytopathic effects of the human immunodeficiency virus (HIV) in vitro. A series of experiments are conducted to compare in vivo effects of the anti-HIV agents with in vitro effects obtained with the same virus. Currently, the project utilizes two murine retroviral models for these studies, the Rauscher murine leukemia virus (R-MuLV) and the LP-BMS MuLV (MAIDS) model. Results from the project will be interrelated with pharmacologic and toxicologic information to devise and recommend treatment strategies for clinical trial. This contract is being recompeted.

SOUTHERN RESEARCH INSTITUTE (N01-CM8-7285)

This contract provides a resource for preclinical 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 intravenous, subcutaneous (bolus and/or infusion) and oral dosing in mice, rats, and dogs; and (4) identification and pharmacokinetic analysis of drug metabolites. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Data obtained in these investigations are used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Preclinical pharmacology studies of potential anti-AIDS compounds 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 (N01-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 second year of a 3-year contract.

SRI INTERNATIONAL (N01-CM0-7333)

This is one of the Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds that 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.

SRI INTERNATIONAL (N01-CM6-7864)

SRI International is one of two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations that are to be investigated in the pharmacological, toxicological, and clinical phases 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. This contract is being recompeted.

SRI INTERNATIONAL (N01-CM7-3715)

SRI International is one of three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations that 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 (N01-CM0-7341)

This is one of the Master Agreement Contractors for the synthesis of a variety of organic and/or inorganic compounds that 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.

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. This contract is being recompeted.

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. This contract is being recompeted.

STARKS ASSOCIATES (N01-CM8-7231)

This contract is for the synthesis of a variety of organic and/or inorganic compounds that have been identified by program for development. Unique compounds from the literature, with reported biological activity, and 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.

STARKS ASSOCIATES, INC. (N01-CM9-7591)

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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

STARKS ASSOCIATES, INC. (N01-CM9-7626)

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 the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

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: (1) water solubility; (2) hydrolytic stability; and (3) 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.

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

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 to: (1) synthesize congeners of synthetic compounds with confirmed activity; (2) design and synthesize prodrugs and other compounds that posess elements of both congener and prodrug; (3) synthesize compounds related to products of natural origin and other related heterocycles; and (4) synthesize antisense nucleic acids. These products may include partial structures of analogs and novel heterocycles.

TACONIC FARMS (N01-CM5-7730)

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

TECHNICAL RESOURCES, INC. (N01-CM8-7249)

This DTP contract provides a program-wide resource for support servicesto 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 National Cooperative Drug Discovery Group (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 (N01-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 (N01-CM9-7585)

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, which is being applied to resolve difficult formulation problems.

VERMONT REGIONAL CANCER CENTER (N01-CM8-7286)

This contract provides a resource for preclinical 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 intravenous, subcutaneous (bolus and/or infusion), and oral dosing in mice, rats, and dogs; (4) identification and pharmacokinetic analysis of drug metabolites. Characterization of oral bioavailability, dose-dependent kinetics, and species differences in kinetic behavior may also be performed. Data obtained in these investigations are used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Preclinical pharmacology studies of potential anti-AIDS compounds 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 contract was responsible for the design, coding, and maintenance of the computer support system for processing the biological data generated by the DTP anti-AIDS and anticancer screening programs. This contract expired December 31, 1989.

WORLD BOTANICAL ASSOCIATES (N01-CM8-7281)

This Master Agreement for Master Agreement Orders in the large-scale recollection of plants for anti-AIDS drugs was established to permit the isolation and purification of anti-HIV-active agents for preclinical and clinical development. 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 recently completed an assignment to recollect thirteen lichen species for further study as sources of potential anti-HIV agents.

Z, INC. (N01-CM7-3720)

The objective of this 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 contract also performs searches for crude natural products on a selected basis.

RADIATION RESEARCH PROGRAM

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

This contract continues to develop 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. The 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 1,200 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-CM9-7602) (SMALL BUSINESS INNOVATION RESEARCH Program)

Analysis and Simulation, Inc., Buffalo, New York, 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 database system, such as the NCI's Physician Data Query (PDQ) database.

MASSACHUSETTS, UNIVERSITY OF (NO1-CM9-7570)

The University of Massachusetts, Worcester, MA, was awarded a contract on June 1989. This was in response to an RFP (NCI-CM-57744-26) entitled, "Single Photon Radiopharmaceuticals for Function, Metabolism and Tissue Localization." The Massachusetts researchers are developing radiopharmaceuticals for Single Photon Emission Computed Tomography (SPECT). SPECT is a promising technology for non-invasive anatomic and functional diagnosis. This contract is conducting research in the designing, synthesizing, labeling and initial testing of radiopharmaceuticals which may be biomedically useful for diagnostic imaging and cancer detection. An aspect of this research will involve the use of monoclonal antibodies as tissue-specific probes for diagnostic imaging.

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

Megavoltage Imaging, Gainesville, Florida, 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 collection projection data with a suitable ionizing liquid and producing an image within a few seconds.

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; 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.

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 50% of the tumors. All radioprotective compounds tested will be compared with the standard WR -2721. This 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.

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 affinic nitroimidazoles. 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 SBIR contract is pursuing two complementary paths of activity in its Phase II program to design, develop, and evaluate a high resolution ultrasound system (scanner) for medical diagnostic imaging of abdominal organs and the breast. Based on the feasibility results of the Phase I test scanner, a more advanced hardware system is being designed and constructed. This work comprises design and procurement of transducer elements for the scanning arrays for organ examination as well as overall system design and construction. At the same time an extensive parallel effort is under way in the design of software (algorithms) to provide the computer-based functions which drive the electronic hardware system in its acquisition and display of medical images. Principles of synthetic focus, inverse scattering, and adaptive arrays will be employed to achieve more accurate cross-sectional ultrasound images of the body than have been previously attainable.

TEXAS, UNIVERSITY OF, SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE) (N01-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. Three clinical studies are currently under investigation by the NTCWG in several anatomical sites: head and neck, prostate and lung.

TEXAS, UNIVERSITY OF, SYSTEM CANCER CENTER (M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE) (N01-CM6-7914)

The University of Texas System Cancer Center, M.D., Anderson Hospital, was a member of the Electron Collaborative Working Group (ECWG) from 1986-89, a consortium of three institutions funded to evaluate electron beam treatment planning. The final report of ECWG contains recommendations and guidelines for the planning, dose calculation, dose measurement, error analysis and new treatment techniques in electron beam radiotherapy.

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)

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SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE BIOLOGICAL RESPONSE MODIFIERS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1989 through September 30, 1990

INTRODUCTION

The Biological Response Modifiers Program (BRMP) is a comprehensive program with both extramural and intramural basic and clinical research components charged with the investigation, development, and clinical testing of biological approaches to cancer treatment. The unique structure of the program facilitates the rapid movement of basic science and preclinical observations into clinical testing.

The extramural research activities sponsored by the BRMP are planned, supervised and monitored by the Biological Resources Branch (BRB) under the direction of Stephen P. Creekmore, Ph.D., M.D. The BRB maintains a large grant portfolio and oversees contracts supporting preclinical and clinical research throughout the biomedical research community. BRB activities include sponsorship of Phase Ia, Ib, and II clinical trials of biological agents that aim to induce antitumor responses and to assess measurable changes in host biological functions that may be associated with antitumor responses. Biological drug development is conducted in close cooperation with the Developmental Therapeutics Program (DTP) of the Division of Cancer Treatment (DCT) and with the Cancer Therapy Evaluation Program (CTEP). A group of individuals comprise of key personnel from the BRMP, DTP, and CTEP form the Biological Response Modifiers Operating Committee (BOC). The BOC focusses on problem-solving and coordination of preclinical and clinical drug development of biological and growth modifying agents. The BOC conducts the entire range of drug development activities from procurement, identification, production, and toxicity assessment, all the way to clinical testing. The BOC, under the direction of Dr. Stephen Creekmore, acts on advice of the DCT Decision Network Committee, which is responsible for allocating drug development resources and prioritizing biological agents for clinical development.

The BRB also supervises an intramural contract-supported preclinical evaluation laboratory that studies the activity and mechanisms of action of biological agents in preclinical animal models with the goal that such studies will have an influence on the design and monitoring of human clinical trials, including the study of dose- and schedule-related variable, the biological and immunological parameters associated with in vivo tumor regression, and the detection of surrogate markers measurable in man that may correlate with the induction of a significant antitumor effect. The BRB serves as liaison with a large number of biotechnology companies and interacts 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 requests for applications and program announcements to stimulate the development of promising areas of scientific inquiry that may lead to improved cancer treatments.

The intramural research program of the BRMP consists of three basic science laboratories (Laboratory of Molecular Immunoregulation [LMI], Laboratory of Experimental Immunology [LEI], Laboratory of Biochemical Physiology [LBP]), one clinical branch (Clinical Research Branch [CRB]), and laboratory research conducted under the auspices of the Associate Director (OAD).

The OAD investigates the control of B- and T-cell development and proliferation. Studies in this laboratory are aimed at studying lymphocyte ontogeny, particularly focussing on events that shape the B- and T-cell repertoire, tolerance induction, and the physiologic basis of genetic immune deficiencies. In addition, studies on the regulation of lymphocyte proliferation are aimed at understanding the basis of lymphomagenesis and the fundamental control mechanisms of cell cycle progression.

The LMI consists of three sections: the Lymphokine Section (LS) headed by Francis W. Ruscetti, Ph.D., the Immunobiology Section (IS) headed by Luigi Varesio, Ph.D., and the Cytokine Molecular Mechanisms Section (CMMS) under William Farrar, Ph.D. The Chief of the LMI is Joost J. Oppenheim, M.D. 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 consists of three sections: the Cellular and Molecular Immunology Section (CMIS) under Howard Young, Ph.D., the Leukocyte Differentiation Section (LDS) with John R. Ortaldo, Ph.D., serving as acting head, and the Experimental Therapeutics Section (ETS) under Robert H. Wiltrout, Ph.D. The Chief of the LEI 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 monocyte, macrophage, and lymphocyte activation; and (5) prepares new probes to analyze cell differentiation and function, especially lymphoid and hematopoietic cells.

The Chief of the LBP is Hsiang-fu Kung, Ph.D. 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 currently without a permanent branch chief and is being managed on an interim basis by Dan L. Longo, M.D., 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 and lymphoproliferative diseases.

EXTRAMURAL PROGRAM ACCOMPLISHMENTS

There are extensive interactions with other programs with similar and potentially overlapping interest to facilitate smooth coordination of the development of biological therapeutic agents and strategies. In addition to the BOC, which coordinates efforts with the DCT, there is also a close working relationship with the Immunology and Tumor Biology Programs of the Division of Cancer Biology and Diagnosis and Centers and with the AIDS program of the National Institute of Allergy and Infectious Diseases.

Grants Program

In FY90, the BRMP supported 103 grants with nearly $29 million, including 72 research projects (R01), 10 program projects (P01), 1 outstanding investigator grant (R35), 2 phase I SBIR (R43), 2 phase II SBIR (R44), 8 merit awards (R37), 1 conference grant (R13), and 6 first investigator grants (R29). The grant portfolio includes an extensive effort to develop and apply monoclonal antibody technology to the treatment of cancer. Active grants are focussing on melanoma, lymphoma, leukemia, breast cancer, colon cancer, and renal cell cancer. 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 ongoing. The grants program is also supporting work to develop therapeutic agents using molecular biology tools, for example, hormone/toxin hybrid molecules developed under BRMP grants are now coming to clinical trial for the treatment of leukemia, lymphoma, and melanoma. Other investigators 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. New grants began in FY90 to study tumor-infiltrating lymphocytes, to develop human antibodies, to use combinations of cytokines to induce differentiation in tumor cells, and to enhance tumor immunity by the genetic manipulation of major histocompatibility antigens.

Requests for Applications (RFAs)

The BRMP has not been very successful at fostering research through the mechanism of RFAs. It has been our experience that the funding score given by special study sections is often substantially poorer than scores given by standing study sections to the same grant. Only four awards were made in FY90 for previously

issued RFAs. Thus, the program favors the Program Announcement mechanism for encouraging the development of particular research topics. Applications are being received on 16 previously issued Program Announcements.

SUMMARY OF BRB ACCOMPLISHMENTS IN FY90

The extramural program has: maintained 26 contracts for testing BRMs in phase I clinical trials; maintained 3 phase I clinical trials contracts to test monoclonal antibodies and other targeting molecules; maintained 4 phase I clinical trials contracts to conduct phase I clinical testing of cytokines and other immune modulators; produced or procured over 300 grams of preclinical and clinical grade monoclonal antibodies in support of NIH-supported investigations; chelated 3 different monoclonal antibodies for clinical studies of imaging and radiotherapy; expanded the distribution of biological standards to include IL1α, IL1β, IL2, IL3, IL4, IL6, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and transforming growth factor-β (TGF-β); awarded $5.3 million for extramural contracts, $2.8 million in clinical trials and $2.5 million for preclinical contracts.

New Extramural Initiatives

The BRB is in the process of developing Requests for Proposals (RFPs) in the following areas: production of antisense oligonucleotides, production of chimeric human-mouse monoclonal antibodies, recompetition of the BRM repository, and establishment of Master Agreements to support innovative investigator-initiated clinical trials involving complex or sophisticated immunological manipulations. In addition, efforts are underway to establish a mechanism to support a centralized facility for performing immunological monitoring of clinical trials in contract facilities that are not capable of doing so. Finally, efforts are underway to develop mechanisms for screening natural products and submitted biologicals for in vitro BRM activity.

Preclinical Evaluation Laboratory

The Preclinical Evaluation Laboratory (PEL) is operated as a contract with Program Resources, Inc. at the Frederick Cancer Research and Development Center. The PEL 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 be able to be translated into a clinical trial in man.

Information from the PEL continues to influence greatly the nature of the clinical trials that are being performed in the CRB. This year the PEL has developed a new biological assay for TGF-β involving the in vitro growth inhibition of tumor cell line M5076. This assay is much simpler than the standard assay which involved the costimulation with epidermal growth factor of proliferation of normal rat kidney cells in soft agar. As we move forward with the clinical study of TGF-β, the existence of a simple and reliable assay procedure will facilitate our clinical trial monitoring capability.

Recent studies on the impact of repeated cyclic use of colony-stimulating factors (CSFs), which mimics how CSFs will be used clinically to ameliorate the myelotoxicity of cyclic combination chemotherapy, have revealed a disturbing level of stem cell depletion. Mice are given six cycles of cyclophosphamide and the myelotoxicity is treated with six cycles of CSFs alone or in combination. The bone marrow of mice who have been through this treatment is then used to serially rescue lethally irradiated mice and in each irradiated recipient, CFU-S, CFU-C and other measurements of hematopoiesis are made. In animals who received G-CSF or GM-CSF, marrow stem cells are exhausted by the third transfer. Normal marrow can rescue through 12-19 transfers. This work raises the specter that some patients treated with CSFs may experience late marrow failure. On the other hand, it appears that the use of IL1 to enhance myeloid recovery is not producing dramatic stem cell depletion. Since our clinical studies with IL1 are suggesting that it may be effective at promoting platelet production as well as granulopoiesis, IL1 with or without G-CSF or GM-CSF may be more effective than any of these CSFs alone.

The PEL has also been interested in the in vivo antitumor effects and hematopoietic effects of bryostatin, a complex macrocyclic compound isolated from a sea animal called Bugula nerititus. This compound is said to activate protein kinase C. Work in the PEL has demonstrated a spectrum of in vitro antitumor activity of bryostatin and the in vivo efficacy precisely parallels the predicted activity based on in vitro testing. That is, those tumors exquisitely sensitive to bryostatin in vitro are also sensitive in vivo and the tumors that are resistant in vitro are resistant in vivo. There is no evidence that host defense mechanisms are activated by bryostatin. So far the bryostatins have not been as effective at promoting in vivo hematopoiesis as in vitro data from May and colleagues at Johns Hopkins would have predicted. However, it may be of some value when used in conjunction with other CSFs. When sufficient quantities of bryostatin become available, the BRMP will be doing a phase I study focussing on patients with lymphoma, the human tumor type that appears most sensitive in vitro.

The PEL has also developed a nude mouse xenograft model to evaluate the efficacy of activated human monocytes as adoptive cellular therapy of cancer. Mice inoculated with human tumors are treated with elutriator purified human monocytes activated with a variety of cytokines with significant prolongation of survival. Once the program has been optimized, we intend to perform a clinical trial to apply this novel form of adoptive cellular therapy to patients with cancer.

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.

Biological Response Modifiers Program Operating Committee (BRMPOC)

The BRMPOC is the steering committee for the BRMP extramural program. It oversees the activities of the BOC and 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 through the activities of the BOC and BRB staff.

BRMP-Sponsored Meetings

The BRMP helped in the organization and planning of a DCT-sponsored workshop entitled "Combining Biological Response Modifiers with Cytotoxics in the Treatment of Cancer: Developing a Rational Approach to a New Therapy" held in Baltimore on March 5-7, 1990. The BRMP cosponsored with CTEP, the Division of Cancer Biology and Diagnosis and Centers, and Janssen Research Foundation a workshop on "Levamisole: Mechanisms of Antitumor Activity" on June 11-12, 1990. The BRMP also sponsored a number of Therapeutic Applications Group meetings to review progress to date on IL8, IL4, IL1, IL3, and IFN-γ. In addition, 45 leading scientists came to Frederick to present their work and interact with the staff of the BRMP.

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 are 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. In addition to the seminar program that brings outstanding scientists to Frederick, BRMP scientists are frequent participants, organizers, and session chairmen at national and international scientific meetings on basic and clinical science.

Office of the Associate Director

Research in the laboratory 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, Jonathan Ashwell, M.D., and Ada Kruisbeek, Ph.D.

Dr. Ashwell was the first to recognize that each T cell has two types of T-cell receptor. The extracellular domains of both types are specific for the same antigen-MHC combined determinants but the intracellular domains lead to distinct signalling machinery. Signalling through the receptor containing a ζ-ζ homodimer leads to tyrosine phosphorylation while signalling through the receptor containing the ζ-η heterodimer leads to phosphoinositide turnover. The ζ-η heterodimer receptor comprises a variable fraction of a cell's T-cell receptors but usually 20% or less. Ashwell has now shown that the level of expression of the ζ-η receptor correlates with the ability of the cell to undergo negative selection or tolerance induction. During ontogeny, the level of expression of the ζ-η receptor increases dramatically between day 16 and 17 after conception, decreases thereafter until day 22 or 23, and then increases to about the 20% level seen in adult T cells by the 3rd week of life. The level of expression of ζ-η correlated precisely with the capacity of the cells to be clonally deleted upon exposure to antigen. It appears from these data that the ζ-η receptor may

regulate the programmed cell death pathway of the developing thymocyte and T cell. Another stimulus that is capable of killing thymocytes and T cells is corticosteroids. Ashwell and his colleagues have shown that these two pathways mutually interfere with one another since signal transduction through the antigen receptor does not lead to cell death in the presence of steroids and steroids do not kill the cell in the presence of its antigen. In addition, Ashwell and his colleagues have found that T-cell activation through the T-cell receptor requires the participation of a cell surface protein called CD45 which is linked to the receptor and serves as a tyrosine phosphatase. The study of the role of the phosphatase in signalling is underway. Dr. Ashwell's work has led to a number of clinical trials in which T-cell neoplasms are being treated with antibodies designed to induce programmed cell death and in which efforts are being made to boost T-cell activity against a tumor by triggering the cells through their T-cell receptor.

Dr. Ada Kruisbeek and her colleagues have been among the world's leaders in dissecting the function of the thymus in T-cell development. Her work has demonstrated an important role for the cytokines IL2 and IL4 in early thymocyte development before the cells express T-cell antigen receptors. Once such receptors are expressed, she and her colleagues have shown that whether the cell is positively selected for expansion or negatively selected for deletion relates to cellular interactions in the thymus in which the receptor and CD4 and CD8 antigens on the thymocytes and class I and class II MHC molecules on the thymic epithelial or stromal cells play a crucial role. Kruisbeek and her colleagues have made a critical breakthrough in understanding the mechanism of these effects through the development of an in vitro thymic organ culture system in which a clonal transformed thymocyte cell line that does not express T-cell receptors can be induced to express its receptor by coculture with the thymus. Studies are underway to dissect the influence of the thymus on the developing T cell. Dr. Kruisbeek has also found that some processes that shape the T-cell repertoire occur extrathymically. By studying T cell in neonatally thymectomized and in nude mice, she and her colleagues have demonstrated that autoreactive T cells can be induced to become anergic in the periphery. Such studies should lead to clinical advances in understanding the recovery of immunity in patients undergoing bone marrow transplantation and in the selective induction of tolerance to particular antigens, e.g. therapeutic xenogeneic antibodies or immunotoxins or even self antigens in patients with autoimmune phenomena.

In collaboration with James J. Kenny, Ph.D. of the Biological Carcinogenesis Development Program, Program Resources, Inc., the laboratory is also evaluating the development of the B-cell repertoire. Using transgenic mice expressing either heavy chain genes, light chain genes, or both, several novel insights have emerged. First, in <u>xid</u> immune defective mice, there is evidence that some of the immune defect is related to antigen-specific clonal deletion. These animals are incapable of responding to certain T-independent antigens such as phosphocholine, a common component of bacterial cell walls. If one inserts into the germline rearranged heavy and light chain genes that encode an intact antibody recognizing phosphocholine, one finds that the immune defective mouse has normal numbers of B cells in the bone marrow, but no phosphocholine-specific cells appear in the periphery. The problem is not related to the transgenes themselves since animals made with only the heavy chain gene are capable of exporting transgene-expressing B cells to the periphery of immune defective mice, but none of the antibodies containing the transgene are specific for phosphocholine. In other words, the assembly of the transgene heavy chain with a variety of endogenous light chains

proceeded normally, but all of those antibodies with specificity for phosphocholine were deleted. This is the first evidence that B cells undergo clonal deletion driven through their antigen receptor as an influence that shapes the B-cell repertoire. The cellular basis for this process in under investigation.

Margaret Beckwith, Ph.D. and Chou-Chi Li, Ph.D. of the Biological Carcinogenesis Development Program, Program Resources, Inc., are collaborating with the Associate Director in an effort to understand the mechanism by which malignant B cells undergo irreversible growth arrest when signals are transduced through the B-cell antigen receptor, namely surface immunoglobulin. Dr. Beckwith has demonstrated that the growth arrest mediated by anti-μ coupled to beads is independent of protein kinase C. However, the process seems to involve signal transduction that is coupled with a novel G-protein that is cholera toxin sensitive but not linked to adenylate cyclase, as most Gs proteins would be expected to be. Study of phosphoproteins induced by signal transduction through anti-μ reveals clear differences between anti-μ and protein kinase C-induced changes. The cell line under study also expresses surface IgD and appears to be growth inhibited by bead-associated anti-δ but growth stimulated by soluble anti-δ. This tumor cell line will permit the dissection of signal transduction through surface IgM and IgD.

One of the most interesting observations on the mechanism of growth arrest of the lymphoma cells mediated by phorbol esters and bryostatin is that they appear to induce an autocrine negative pathway mediated by TGF-β. In collaboration with Dr. Francis Ruscetti, it was found that the normal proliferating lymphoma cells produce only small amounts of latent TGF-β and do not express receptors for TGF-β. Under the influence of picogram amounts of phorbol esters, the cells are induced to express TGF-β receptors and active TGF-β and the phorbol ester-induced growth inhibition is reversible by antibody to TGF-β. This is the first demonstration of growth regulation in a malignant cell by induction of an autocrine growth inhibitory pathway.

Dr. Chou-Chi Li has been interested in the expression of the mos oncogene in human tumor cells. This oncogene is also known as meiosis promoting factor and it plays a role in stimulating a cell to divide. Mos has only been identified in murine germ cells and has been difficult to demonstrate in either normal or neoplastic human tissue. Using the PCR technique and a sensitive antibody, she has now shown that mos mRNA and protein are more widespread than previously thought. She has found both RNA and protein in human neuroblastoma cell lines and cervical cancer cell lines. In view of the role of this oncogene in cell proliferation, it may be a target whose blockade will lead to growth arrest.

Douglas K. Ferris, Ph.D. and Chou-Chi Li, Ph.D., of the Biological Carcinogenesis Development Program, Program Resources, Inc. are collaborating with the Associate Director on the control of cell cycle progression. They have identified a tyrosine kinase that inactivates the cdc2/p34 "master kinase" in a cell cycle dependent fashion and are examining growth arrested cells to see if the cell cycle block is related to the inactivation of cdc2/p34. Dr. Ferris is also examining the role of a new nuclear tyrosine kinase oncogene called fer in cell proliferation. Using antisense oligonucleotides, Dr. Ferris has shown that the synthesis of fer protein can be blocked and in such cells proliferation ceases and there is some evidence that differentiation begins. Further studies of the function of this oncogene are in progress.

Laboratory of Molecular Immunoregulation (LMI)

The LMI investigates at a molecular level the intercellular and intracellular processes that regulate host defense mechanisms. These studies are conducted in three sections and in the office of the chief. The research conducted in the office of the chief is carried out by three tenured senior investigators, Joost J. Oppenheim, M.D., Kouji Matsushima, M.D., and Scott K. Durum, Ph.D.

Dr. Joost Oppenheim is an internationally renowned and award-winning immunologist who has contributed significantly to our understanding of IL1 biology and action. Most recently he has turned some of his attention toward the clinical application of IL1 to the treatment of patients with cancer. He and his colleagues have shown that the administration of IL1 to mice together with bone marrow cells after lethal irradiation improves the capacity of limited numbers of cells in the bone marrow inoculum to repopulate the recipient. Thus, pretreatment of animals with IL1 protects the marrow against the lethal effects of radiation and administration of IL1 with marrow after irradiation aids in reconstitution. IL1 is both a radioprotector and a colony-stimulating factor. Dr. Oppenheim has also studied the role of cytokines in the promotion or prevention of neoplastic transformation. Using a non-tumorigenic murine epidermal cell line called JB6, he has found that stimulation of the cell with TGF-α blocks the phorbol ester-induced neoplastic transformation of the cell, an effect that can also be mediated by retinoids. However, the mechanism of action of retinoids and TGF-α appear to be distinct since their use together is synergistic in its anti-transformation effects. In contrast, TNF-α promotes the transformation process and synergizes with phorbol ester. This is the first demonstration of the potential tumor promoting effects of cytokines.

Dr. Kouji Matsushima and his colleagues have made impressive progress in understanding the effects IL1 exerts on the cells it stimulates. One of the early events is the activation of a novel serine kinase that is distinct from protein kinase C, protein kinase A, and other known serine kinases. The major cell substrate of the kinase is plasmin. The novel kinase is also activated by TNF-α, which shares many activities with IL1. In addition to the cytoplasmic events that occur upon exposure to IL1, Matsushima and his colleagues have also characterized an IL1-responsive region on a number of IL1-induced genes. They are on the threshold of characterizing the entire signal transduction pathway from cell surface binding to gene activation. In addition to this prodigious accomplishment, Dr. Matsushima and his colleagues have identified, cloned, sequenced, and expressed two novel cytokines, IL8, a member of a family of low molecular weight growth factors that activates and chemoattracts neutrophils and chemoattracts T cells, and MCAF, a monocyte activating and chemotactic factor. Both these molecules are important mediators of inflammatory responses and both have potential clinical applications.

Dr. Durum and his colleagues have been studying the role of IL1 and antigen as costimulators of T cells. Signalling through the T-cell receptor induces expression of the fos oncogene and IL1 induces the expression of the jun oncogene and together these two oncogene products form the AP-1 transcription factor that acts to stimulate the activation of the host of genes that characterize the T-cell activation response. In other IL1-responsive cell types that do not require costimulation, IL1 stimulates the production of both fos and jun without additional stimuli. Durum and colleagues have also elucidated some of the mechanisms that regulate the rearrangement of genes encoding the T-cell receptor

for antigen. Using a PCR technique, he and his colleagues have shown that the physical rearrangement of the genes occurs even before the T-cell precursors arrive in the thymus. The processes that regulate the ordered expression of the genes after rearrangement during ontogeny are under investigation.

Luigi Varesio, Ph.D., is the head of the Immunobiology Section of the LMI. Varesio and his colleagues have found that human monocytes constitutively express the p75 IL2 receptor and that stimulation of monocytes with IL2 leads to the expression of the p55 IL2 receptor subunit, c-fms expression (the M-CSF receptor), and the development of cytotoxicity directed against tumor cells. Once c-fms is expressed, exposure of the monocytes to M-CSF prolongs their ability to kill tumor cells. In contrast, IFN-γ also activates monocytes to become cytotoxic but the pathway is clearly distinct from that stimulated by IL2. IFN-γ actually blocks the expression of c-fos, which is induced by serum and by IL2. In addition, it appears that some of the cytotoxicity induced by IFN-γ is related to a product of tryptophan metabolism called picolinic acid. Picolinic acid by itself stimulates many of the functions that are stimulated by IFN-γ and may be a novel BRM with potential clinical application. Varesio and his colleagues are also studying the mechanisms by which HIV expression is regulated in monocytes. They have identified a nuclear factor that is inducible by lipopolysaccharide and binds to the LTR of HIV resulting in the activation of HIV transcription. The appropriate combination of monocyte activating agents to induce HIV production together with antiretroviral therapy might lead to the eradication of monocytes as a reservoir for HIV.

Francis Ruscetti, Ph.D., is the head of the Lymphokine Section of the LMI. Dr. Ruscetti is a world authority on the control of hematopoiesis and retrovirology. He is the codiscoverer of IL2 and of HTLV-I. His studies on the role of cytokines in hematopoiesis have led to the finding that TGF-β reversibly blocks primitive hematopoietic stem cells from proliferating and at the same time synergizes with GM-CSF to promote granulopoiesis. TGF-β leads to the down-regulation of receptors for the colony-stimulating factors on the surface of stem cells. Studies are underway to evaluate the signal transduction mechanisms that lead to altered receptor gene expression. Together with Dr. Robert Wiltrout, efforts are underway to examine the in vivo effects of TGF-β on hematopoiesis. Preliminary data suggest that TGF-β may be useful in protecting the stem cell against the toxicity of chemotherapy and perhaps radiation. TGF-β may exert antitumor effects on certain leukemic cell lines as well.

Dr. Ruscetti and his colleagues are also studying the mechanism by which cells regulate the expression of human retroviruses, HTLV-I and HIV. Dr. Ruscetti and his colleagues have found that B cells infected with HTLV-I produce a factor that blocks the transactivation of viral replication. Even when a construct containing an active transactivator is transfected into B cells, some host cell gene product blocks viral replication. Efforts are underway to identify this suppressor of transactivation. HIV latency in monocytes may represent a related phenomenon. In one form of HIV latency, control of viral replication in monocytes appears to be related to the methylation of the HIV LTR prohibiting the viral promoter from being activated. Demethylation with 5-azacytidine reverses this form of latency. In the second form of latency, it appears that there are nuclear factors in latently infected cells that prohibit the formation of the active NFκB heterotetramer that promotes HIV transcription. This nuclear factor that blocks NFκB could be related to IκB. Further studies are necessary to identify this transcriptional regulatory protein.

William Farrar, Ph.D., is the head of the Cytokine Molecular Mechanisms Section of the LMI. Dr. Farrar's major area of investigation has been to elucidate the mechanisms by which cytokine receptors that are devoid of intrinsic kinase activity, such as IL2, IL3, and GM-CSF receptors, nevertheless mediate protein phosphorylation as an integral component of their activation of target cells. Using a variety of innovative techniques, Farrar and his colleagues have isolated the IL2 receptor together with a complex of proteins with which it is associated. It appears that one of the associated proteins, p97, is a tyrosine kinase that may mediate IL2-driven activation. Efforts to purify, sequence, and clone the IL2 receptor-associated tyrosine kinase are underway. In related work, Farrar's group has cloned 5 new tyrosine kinases from an expression library derived from human leukemic cells. Efforts are now underway to analyze what role these kinases may play in normal and leukemic cell proliferation.

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. John Ortaldo, Ph.D., is the Chief of this laboratory.

The Leukocyte Differentiation Section (LDS) has Dr. John Ortaldo as its acting head. It studies the differentiation and activation of human and murine lymphocytes and the role of the cellular immune system in mediating antitumor responses. This research group has made the remarkable discovery of the putative NK receptor, the cell surface protein on NK cells responsible for the recognition of tumor cell targets. Using an ingenious strategy, they made antibodies to an NK target cell, K562, which blocked target recognition by NK cells. They then made anti-idiotypic antibodies to antibody that recognized the target and the anti-idiotype recognizes NK cells and blocks binding and lytic functions of NK cells. The anti-idiotypic antibody reacts with an 80-100 kD and a 160 kD protein. The target has been molecularly cloned in collaboration with Dr. Howard Young of the Cellular and Molecular Immunology Section of LEI and the gene has been mapped to the long arm of chromosome 3, a site commonly deleted in renal cell cancer, small cell lung cancer, and some other tumor types. It is not clear whether NK function is altered in patients with these tumors or whether the 3p deletion affects the capacity of the host to recognize the developing tumor.

In other studies aimed at identifying cellular mechanisms involved in target cell lysis, the LDS investigators have characterized a granule protein called NK cytotoxic factor or NKCF, a 12,000 MW protein that is distinct from other known cytokines including TNF and lymphotoxin. Using antibodies to NKCF, they have successfully blocked NK activity, suggesting that NKCF is the principal mediator of NK activity. One of the major contributions of this research group under Ortaldo has been its demonstration that the NK cell or CD3-negative large granular lymphocyte is an important immunoregulatory cell in that it secretes a large number of cytokines including IL1α, IL1β, IL2, IFNα, IFN-γ, IL4, GM-CSF and B-cell growth factor. These cells respond to IL2 by producing a variety of cytokines and broadening their tumor target cell specificity. Ortaldo and his colleagues have shown that the CD3-negative large granular lymphocytes express only the p75 intermediate affinity IL2 receptor. Many of the activation functions exerted on LGL by IL2 are purely through the p75 receptor. On the other hand, over time after IL2 stimulation, p55 IL2 receptor expression is induced and when this occurs, the cells proliferate in response to IL2. Thus, IL2 induces two phases of activities, first activation and second proliferation.

Investigators in the LDS have also been studying the ontogeny of LGL and made the surprising finding the liver-associated LGL appear to be derived from dull Ly1-positive thymocytes, since reconstitution of LGL in a lethally irradiated animal can proceed when dull Ly1-positive thymocytes are the cell population adoptively transferred to such hosts. In addition, the LDS group has developed an antibody to murine NK cells that subsets these cells into two nonoverlapping subgroups: LGL-1-positive cells mediate NK activity but LGL-1-negative cells can be activated to express LAK activity by IL2.

The Cellular and Molecular Immunology Section (CMIS) under Howard Young, Ph.D., 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 may be enhanced. Dr. Young and his colleagues have been interested in the regulation of IFN-γ gene expression which normally occurs in only 2 cell types, T cells and LGLs. Young and colleagues have found that IFN-γ expression is under the influence of at least two enhancers, one of which is tissue-specific. The second enhancer appears to be under the influence of calcium flux. Recently these investigators have found IFN-γ to be aberrantly expressed in two B-cell tumor lines after induction with protein kinase C activators. They believe that the study of these lines will reveal insights on what normally negatively regulates the gene.

The CMIS has also been conducting studies in conjunction with Dr. Robert Wiltrout in the Experimental Therapeutics Section (ETS) to dissect the mechanism by which flavone 8-acetic acid (FAA) produces immune stimulation in mice. They have shown a particular sequence of induction of cytokine genes in murine spleen cells. Efforts to reproduce the antitumor effects of FAA by replicating the same sequence of cytokines administered systemically have partially succeeded; however, the induction of endogenous cytokine production appears to be a much more efficient means of inducing an antitumor effect in animals.

As mentioned above, CMIS investigators have been instrumental in the collaboration that has led to the cloning and molecular characterization of the NK receptor for tumor cells. Sequence data indicate an unusual protein with an external domain that has homology to cyclophilin, the intracellular target of cyclosporin A that has cis-trans isomerase enzymatic activity. Its extracellular role can only be hypothesized at this point. In addition, CMIS scientists have been studying the regulation of expression of the perforin gene. Though perforin mRNA is constitutively expressed in LGL, it is not inducible by any activation stimuli and it does not appear to play an important role in LGL function. On the other hand, peripheral blood CD8-positive T cells can be induced to express large amounts of perforin mRNA by exposure to IL2. Furthermore, the amount of IL2 required to induce perforin expression is reduced nearly 2 logs by the costimulation of the T cells with IL6. Thus, IL6 plus IL2 represent a potent stimulus to the development of cytotoxic activity in T cells and may be exploitable in man to treat cancer.

Robert H. Wiltrout, Ph.D., is the head of the Experimental Therapeutics Section (ETS) of the LEI. This section studies the antitumor efficacy and mechanisms of action of biological agents effective in the treatment of animal tumors in vivo. Wiltrout and his colleagues have dissected the basis of the in vivo antitumor effect of FAA plus IL2 against both renal and colon cancer in mice. FAA is a potent inducer of cytokine production in mice. As a direct result of experiments

performed by Wiltrout and colleagues, a clinical trial of FAA and IL2 was performed; however, the human clinical trial failed to demonstrate in vivo cytokine induction by FAA in man. The ETS analyzed potential reasons for the differences and found that administering FAA, a weak acid, with urinary alkalinization, as is done in man to prevent crystallization in the urine, neutralizes the effects of FAA on cytokine induction. The clinical trial accordingly was modified to see if patients receiving FAA without alkalinization may experience the cytokine induction seen in animals.

Animals cured of tumors by the combination of FAA and IL2 have been shown to be immune to rechallenge. Wiltrout and colleagues have demonstrated that animals treated with FAA and IL2 develop CD8-positive T-cell immunity directed against the tumor. Furthermore, depletion of the CD8-positive T-cell subset during treatment also blocks the therapeutic effect. Thus, FAA and IL2 are acting as true biological response modifiers in that direct antitumor activity is insufficient to produce tumor regressions.

ETS scientists have also studied the mechanism of the antitumor effects of LAK plus IL2. They have found that IL2-stimulated NK cells traffic poorly to tumor sites but produce abundant cytokines in vivo. The antitumor effects appear to be due either to direct tumor killing by cytokines secreted by the LAK cells or alternatively that the cytokines stimulate host T cells and monocytes to become cytotoxic.

John W. Pearson, Ph.D. and colleagues in the ETS have demonstrated a remarkable synergistic antitumor interaction by administering IFNα together with an immunotoxin. The antitumor synergy has been seen with both holotoxins (i.e. antibodies linked to Pseudomonas exotoxin) and hemitoxins (i.e. antibodies linked to ricin A chain) and has been seen both in vitro and in vivo. Nude mice with established human tumor xenografts of colon and ovarian cancer can achieve nine logs of tumor reduction with combination chemotherapy followed by immunotoxin plus IFNα. Those mice that are not cured by such therapy appear to have small residual solid tumor deposits but such cells do not express any in vitro toxin resistance, drug resistance, or alteration in expression of the target antigen. Similar synergy has been seen with IFN-γ plus immunotoxins and in vitro synergy has been shown with cyclosporin A and other calmodulin antagonists.

ETS scientists are also investigating the appearance of activated lymphoid cells in the liver after administration of a BRM in vivo to mice. The cells that appear in the liver appear to be called up from the bone marrow and can respond chemotactically to factors produced by hepatocytes and Kupffer cells after BRM administration. Studies are underway to identify these hepatic chemotactic factors. In collaboration with Thomas Sayers, Ph.D. of the Biological Carcinogenesis Development Program, Program Resources, Inc., ETS investigators have also characterized what appears to be a novel tumor growth inhibitory cytostatic factor from large granular lymphocytes. The novel factor is 32kD and appears distinct from any previously reported cytokine. Efforts are underway to clone the factor.

Wiltrout and colleagues have also been conducting experiments on the in vivo effects of TGFβ on hematopoiesis. They have shown that TGFβ can transiently arrest the proliferation of early hematopoietic stem cells in normal mice and in those whose stem cells have been recruited into cycle after administration of 5-fluorouracil. In both systems, in vitro assay of marrow from TGFβ-treated mice

revealed a 50% reduction in colony-forming activity. ETS researchers have also used IL1 for the first time to dose-escalate myelotoxic chemotherapy in tumor-bearing mice. They found that IL1 allowed a higher and more effective dose of cyclophosphamide to be administered; however, animals died of late pulmonary toxicity that was not protected against by IL1. These important experiments illustrate two important points: first, it may well be possible to use currently available agents at higher and more effective doses; second, such augmented doses may replace one dose-limiting toxicity (in this case, myelotoxicity) with another. We know very little about the second organ toxicity that becomes dose-limiting for most chemotherapeutic agents in man since our phase I toxicity testing is aimed at determining only the most sensitive organ.

Laboratory of Biochemical Physiology

The Laboratory of Biochemical Physiology (LBP) under the direction of Hsiang-fu Kung, Ph.D. performs studies on the genetic and biochemical events related to the development and expression of the malignant phenotype. LBP has focussed its studies on the biochemical mechanisms of signal transduction pathways and has applied their studies both to factors involved in tumor cell proliferation and in the response of cells of the immune system to their growth factors and stimulatory cytokines.

Dr. Kung and his colleagues are among the world's leaders in research on the function of ras oncogenes. The ras gene encodes for membrane-associated protein of 21kD molecular weight that binds guanosine triphosphate (i.e. G-protein). A single point mutation can render the cellular ras gene product into a transforming factor. Kung and his colleagues have performed site-specific mutagenesis to dissect the functional domains of ras proteins and have elucidated much about their regulation in addition to their structure-function relationships.

Kung and colleagues have clarified a troubling enigma about ras function. Normally, ras protein is inactive when it is associated with guanosine diphosphate (GDP) and active when associated with guanosine triphosphate (GTP). Purified ras seems incapable of getting rid of GDP and picking up GTP. They have identified a 35kD factor that exchanges GTP for GDP (i.e. pulls GDP off inactive p21 and replaces it with GTP) and enhances the function of ras. This new guanine nucleotide exchange factor or GEF may be a target for regulation of ras activity and may be a novel example of an oncogenic protein.

It has previously been shown that ras is associated with another protein that activates the GTPase activity of ras. This factor is called GTPase activating protein or GAP. Kung and colleagues have shown that this factor has important activities independent of its association with ras in that microinjection of ras, GAP, and antibody to ras still results in the generation of diacylglycerol, which activates protein kinase C. Thus, GAP acts to phospholipid breakdown and provides a novel pathway of regulation of the phospholipid-dependent protein kinases, which are critical components of signal transduction pathways.
Ras is a member of a family of proteins called small molecular weight G-proteins or smg. Another member of this family is ADP-ribosylation factor, an smg associated with the large molecular weight G-protein, Gs. Kung and colleagues have cloned human and Xenopus ARF and have found its sequence to be remarkably conserved. ARF inhibits ras-induced and insulin-induced oocyte maturation, and

thus, may play a role in antagonizing ras-mediated cellular processes, perhaps including proliferation. Thus, the mechanism of ras antagonism by ARF is under further study.

The role of protein kinase C in ras-mediated cell transformation is also under investigation. Kung and colleagues have shown that microinjected protein kinase C potentiates ras protein action by increasing the rate of induction of ribosomal S6 protein phosphorylation and speeding oocyte maturation. The basis for the synergistic effect of protein kinase C on ras is not yet clear. Ras is also interrelated with the effects of other oncogenes. For example, antibody to the ras protein can block transformation mediated by fes, fms, and src, but does not affect mos and raf transformation. Raf and mos may act downstream of ras or via an independent pathway. Raf protein was purified and molecularly cloned. Microinjection of raf stimulated DNA synthesis in quiescent cells. Recently, neutralizing raf antibodies were developed in the laboratory and will be used to further dissect the mechanism of raf action.

Kung and his colleagues have also recently demonstrated that eukaryotic translation initiation factors (eIFs) can control cellular proliferation by a mechanism that is not yet clear. They microinjected eIF-4E or eIF-4F into quiescent cells with the result that DNA synthesis was stimulated and the cells took on the morphology associated with the transformed phenotype. This novel finding places emphasis on translation control as another site of action of potentially transforming oncogenes.

Michael Schwabe, M.D. in Kung's group has found that, analogous to IL2, there appears to be a second IL6 receptor with a higher affinity for IL6 than the originally described 80kD receptor. This new IL6 receptor is 106kD and may be a more important target for inhibition of myeloma cell proliferation than the 80kD receptor. They have also shown that IFNα downregulates IL6 receptors on myeloma cell lines, an action that may be the mechanism of interferon's antitumor effects in myeloma in man.

Dr. Kung and his colleagues have also been actively engaged in research on the replication of HIV in monocytes. Using Southwestern screening, they have isolated genes for several previously unknown DNA binding proteins that bind to the HIV LTR at specific sequences and appear to inhibit its replication. In addition, Kung's group is searching for novel antiretroviral compounds purified from Chinese medicinal plants. One such protein called MAP-21 has been shown to exhibit dose-dependent inhibition of HIV infection and replication as measured by inhibition of syncytium formation, reduced in vitro p24 expression, and reduced reverse transcriptase enzymatic activity. MAP-21 was active at concentrations that were neither cytocidal nor cytostatic for the infected cell line. Surprisingly, MAP-21 bears homology to ricin A chain and the trichosanthins. Two other promising antiretroviral natural products have been isolated from two other plants. Their characterization is just beginning.

Clinical Research Branch

The Clinical Research Branch (CRB) conducts treatment protocols for a variety of human cancers. The staff of the CRB has special expertise in the use of biological agents and the treatment of lymphomas and Hodgkin's disease. It 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 operates a 13-bed inpatient unit that contains a 4-bed intensive monitoring unit plus a 3-bed cytapheresis unit in the Frederick Memorial Hospital and a 12,000 square foot outpatient clinic located at the Frederick Memorial Hospital Cancer Treatment Center across the street from the hospital.

In the past year, CRB has continued to explore the uses of IL2 alone and in combination with chemotherapy, other biological agents, and adoptive cellular therapy. Early clinical studies with IL2 suggested that administration of IL2 that led to the induction of peripheral blood LAK activity were associated with tumor responses in patients with melanoma. CRB staff has devised a method of delivering IL2 twice weekly by 24-hour infusion that elicits very high levels of peripheral blood LAK activity in 100% of patients. Unfortunately, the response rate was not enhanced by this achievement. In an effort to overcome potential suppressor cell activity, we administered IL2 preceded by cyclophosphamide based on abundant data from animal models showing improvement in efficacy. The addition of cyclophosphamide did not alter IL2 immunomodulating properties, toxicity, or antitumor effects. Similarly, polyICLC, the standard positive control in animal experiments seeking to elicit a host antitumor response, failed to augment the immunomodulatory activity or antitumor efficacy of IL2. In addition, the encouraging results with FAA plus IL2 in mice were not evident in humans even after testing multiple doses and schedules and modeling the administration based on pharmacological information on FAA levels.

However, one encouraging addition to IL2 was seen. Since large numbers of activated LGLs expressing Fc receptors are induced by certain schedules of IL2 administration, CRB staff added a monoclonal antibody called R24, specific for a melanoma antigen (GD3) and capable of binding to human Fc receptors, to IL2 in patients with melanoma. Four of the first 9 evaluable patients have had partial responses.

In an effort to devise an outpatient combination cytokine regimen, IL2 and IFNα were administered to patients with melanoma. The response rate was not appreciably higher than that obtained with either agent alone. IL2 and IFNα were combined with high-dose cisplatin in a highly toxic inpatient regimen. Four responses were seen in 8 evaluable patients; however, the GI and renal toxicity were prohibitive. Efforts to build on this regimen will include adding ondansetron for emesis control and reducing the dose of cisplatin while adding carboplatin to reduce renal toxicity.

In an effort to improve upon the response rate to LAK plus IL2, CRB tested a combined modality regimen using cyclophosphamide and doxorubicin chemotherapy before LAK plus IL2 and IFNα therapy following LAK plus IL2. The response rate was 20% in both renal cell cancer and in melanoma. In light of the successful early experience adding R24 to IL2 alone, CRB staff is also conducting a protocol to evaluate the efficacy of adding R24 to LAK plus IL2.

CRB has determined an optimal immunomodulatory dose (OID) of IFN-γ in patients treated in the adjuvant setting as well as patients with metastatic disease. Both groups of patients had an OID at 100 mcg/sq M qod. Phase II studies underway in extramural centers will now compare the efficacy of IFN-γ at its OID and its maximal tolerated dose to see whether optimizing its immunologic effects translates into better response rates.

In light of the somewhat surprising efficacy of levamisole plus 5-fluorouracil as adjuvant therapy for stage C colon cancer, CRB made an effort to evaluate the immunomodulatory properties of various doses and schedules of levamisole in the hopes of optimizing its use as compared to the empirically derived dose and schedule used in the large positive clinical studies. Accrual to this study has been completed and data are being analyzed.

The CRB has led the way in the clinical development of IL1α, this year completing the first clinical trial that determined the maximum tolerated dose and also measured immunologic and hematologic effects of the agent. A phase II dose of 0.1 mcg/kg was determined on the basis of hypotension and renal dysfunction at higher doses and a phase II study in melanoma has been started. Dose-related increases in granulocyte count were noted and bone marrow cellularity was increased by IL1. There was also some evidence of increases in platelet counts. A study exploring the myelorestorative effects of IL1 has been started in patients with non-small cell lung cancer treated with carboplatin. A phase I clinical trial of IL1β is also underway.

The CRB is testing other agents that may permit more dose-intense chemotherapy to be administered. GM-CSF is being used to protect ovarian cancer patients against carboplatin-induced myelotoxicity. In addition, efforts are being made to administer MOPP chemotherapy with greater dose-intensity to patients with advanced stage disease and B symptoms, whose probability of long-term survival with standard MOPP is about 60%. Among the first 6 patients to receive dose-intense MOPP, 5 are complete responders and none has relapsed. The MOPP has been given with increased dose intensity but also with greater than usual toxicity. Although granulocytes are favorably affected by GM-CSF, thrombocytopenia is still severe and dose-limiting. There is also interest in GM-CSF as a potential BRM. Patients with intraperitoneal tumors are receiving GM-CSF either alone or combined with IFN-γ or IL2.

Animal experiments conducted in the BRMP have suggested that activation of T cells through the T-cell receptor (e.g. anti-CD3) could be effective at killing T-cell tumors that express CD3 and could activate normal T cells to kill autologous tumor in vitro and in vivo. Patient accrual in T-cell malignancies has been slow but a phase I immunomodulatory study of anti-CD3 has been completed and has determined a dose and schedule to be tested in conjunction with IL2 in an upcoming phase II study. The toxicity from anti-CD3 was unexpected. Dose-limiting toxicity was related to the development of severe headache with evidence for aseptic meningitis in some patients. The basis of this toxicity is not yet understood. Another application of anti-CD3 is in the in vitro activation of peripheral blood T cells. Patients undergo pheresis and have their peripheral blood mononuclear cells incubated for 24 hours with anti-CD3 plus IL2. The cells are then administered back to the patient together with bolus and continuous infusion IL2. This has resulted in a massive expansion of activated T cells in the first 3 patients. It is too early to assess whether the dramatic immune stimulation will lead to an antitumor effect.

The CRB interest in lymphoproliferative diseases has produced a number of disease-oriented studies in addition to the modality-oriented studies noted above. A large cohort of hairy cell leukemia patients has been treated continuously with IFNα for over 5 years. The evidence is that patients continue to have an improvement in their response status with continuous IFN treatment since IL2 receptor levels, which CRB originally described as a tumor marker in

hairy cell leukemia, continue to fall towards normal even when persistent hairy cells can be detected in marrow samples. Some patients developed progressive disease while on IFN. All of these patients had developed a neutralizing antibody to the IFN species used to treat them; however, the antibody was specific for that IFN and did not neutralize other species of IFN. Furthermore, the neutralizing IFN antibody was produced only transiently. Once the antibody disappeared, responsiveness to IFN returned. One rare complication from IFN therapy is the development of erythrocytosis, which we observed in two patients and which contributed to the death of one patient from a bowel infarction. All fifteen of our patients treated with a fourteen month course of alternating deoxycoformycin (DCF) and IFNα are in stable partial response for a median of nearly 3 years off all therapy. Although data on duration of DCF response are sparse, it appears that this alternating combined modality therapy has produced appreciably longer responses than would have been expected with either DCF or IFNα alone. In addition, the dramatic decrease in T-cell number that we initially described related to the DCF appears to be resolving in patients off therapy for 2 years or longer. The low levels of CD4-positive T cells reached levels comparable to AIDS patients but the DCF-treated hairy cell leukemia patients did not suffer an increased incidence of opportunistic infections, as might have been expected.

The major question in the treatment of advanced stage aggressive histology lymphoma is whether more dose-intense chemotherapy will result in a higher cure rate. The CRB is nearly finished with a pilot study of short-course ProMACE-CytaBOM which delivers the 8 drugs at a rate 25-50% greater than conventional ProMACE-CytaBOM. In light of the acceptable toxicity and response rate, the CRB intends to embark on a large prospective randomized trial of ProMACE-CytaBOM vs short course ProMACE-CytaBOM stratifying for known prognostic factors.

The major question in the treatment of the indolent lymphomas is whether aggressive treatment is better than conservative treatment. The CRB is conducting a prospective randomized trial addressing this question. With a median follow-up of 6 years, there is no significant difference in survival between patients treated conservatively and those treated aggressively. However, most of the patients on the conservative arm have been living with active lymphoma. In contrast, only 1/3 of the aggressively treated patients have relapsed and over half have been continuously free of disease for over 7 years. This is clearly better than the 2-year median disease-free survival that had been seen before. This success makes us interested in trying to further improve the therapy of patients with this form of lymphoma with adjustments in the chemotherapy that drop out some of the more toxic agents (e.g. procarbazine) and add fludarabine, an agent recently shown to have significant single-agent activity in indolent lymphoma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09290-05 OAD

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BTS, NCI
Others:	M. Mercep	Visiting Associate	OAD, BTS, NCI
	C. Zacharchuk	Medical Staff Fellow	OAD, BTS, NCI
	S. Volarevic	Visiting Fellow	OAD, BTS, NCI
	C. Burns	Visiting Fellow	OAD, BTS, NCI
	B. Niklinska	Visiting Fellow	OAD, BTS, NCI

COOPERATING UNITS *(if any)*
OAD, BTS, NCI, Bethesda, Maryland (A. Kruisbeek); LAC, NIDDK, Bethesda, MD (S. Simons); Merck Sharp & Dohme, Rahway, NJ (F. Dumont)

LAB/BRANCH
Office of the Associate Director

SECTION
Biological Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	.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.)*

T-cell hybridomas undergo programmed cell death (apoptosis) when stimulated with reagents that are normally mitogenic. Previous work has shown a correlation between this phenomenon and the expression of the T-cell receptor (TCR) $\zeta\eta$ heterodimer. Since immature thymocytes are a normal cell population that regularly undergoes programmed cell death, $\zeta\eta$ expression in these cells was examined. $\zeta\eta$ was found to be highly regulated during fetal ontogeny, increasing dramatically between day 16 and day 17 after conception, steadily decreasing thereafter until day 22 or 23, and finally increasing toward adult levels 3 to 4 wks after birth. This variation in $\zeta\eta$ correlated well with the susceptibility of these cells to negative selection. Using either anti-T-cell antigen receptor antibodies or the SEB "superantigen", it was possible to specifically delete immature thymocytes on day 19, but not on day 26, after conception. Anti-TCR-mediated deletion of thymocytes was found to specifically eliminate those cells expressing relatively high levels of $\zeta\eta$. These data raise the possibility that $\zeta\eta$ may regulate the programmed cell death pathway or be co-regulated with genes that subserve this function. We have found another molecule of physiological importance, the transmembrane tyrosine phosphatase CD45, is physically associated with the T-cell receptor. Mutant T cells that lack CD45 expression are markedly defective in receptor-mediated signally. Effects to transfect the CD45 molecules back into these mutants are underway. The study of T-cell programmed cell death was extended to glucocorticoids. A surprising observation was that the combination of cellular activation and glucocorticoids, rather than being additive or synergistic, was completely ineffective at killing T-cell hybridomas. This effect was reproduced in normal T-cell clones. Activation did not alter steroid-induced translocation of the steroid receptor to the nucleus, nor did it interfere with the glucocorticoid-dependent transcription of a reporter gene controlled by a GRE. The possible physiological relevance of the antagonism between cellular activation and steroid-induced programmed cell death is being explored.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Investigator	OAD, BTS, NCI
Mladen Mercep	Visiting Associate	OAD, BTS, NCI
Charles Zacharchuk	Medical Staff Fellow	OAD, BTS, NCI
Sinisa Volarevic	Visiting Fellow	OAD, BTS, NCI
Christopher Burns	Visiting Fellow	OAD, BTS, NCI
Barbara Niklinska	Visiting Fellow	OAD, BTS, NCI

OBJECTIVES

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

(2) To understand how transmembrane molecules other than the T-cell antigen receptor participate in signal transduction and cellular activation.

MAJOR FINDINGS

I. The TCR in Thymic Ontogeny

The T-cell antigen receptor (TCR) is a complex multi-subunit structure that contains an antigen-binding variable heterodimer ($\alpha\beta$) and associated invariant chains (CD3-γ, -δ, and -ϵ, ζ and η) that transduce signals. TCR ζ generally is found as a ζ-ζ homodimer (ζ_2), but in 5 to 10% of the TCR complexes ζ is found as a heterodimer with the η chain ($\zeta\eta$). We have previously found a correlation between expression of the TCR $\zeta\eta$ heterodimer and susceptibility of an antigen-specific murine T-cell hybridoma, 2B4.11, to activation-induced programmed cell death (apoptosis). To determine if this heterodimer might have functional importance in a physiological setting, we have studied the expression of $\zeta\eta$ in immature thymocytes that must undergo negative selection in the thymus. The quantitation of ζ_2 and $\zeta\eta$ was done by separating the proteins of whole cell lysates by 1-dimensional SDS-PAGE, transferring the material to nitrocellulose, and immunoblotting with an anti-ζ/η antiserum (kindly donated by Dr. David Orloff). Extensive analysis of thymocytes from day 16 after conception to adult mice revealed the following: on day 16 very little ζ_2, and no $\zeta\eta$, is detectable. On day 17, ζ_2 levels are at their apex (~10 to 20 times higher than on day 16), and $\zeta\eta$ is easily detectable. From day 18 to day 23 there is an inexorable decrease in both ζ_2 and $\zeta\eta$. Interestingly, despite the fact that the quantity of both TCR-associated chains decreases over this time, there is also a change in the ζ_2:$\zeta\eta$ ratio of about 3-fold (the ζ_2:$\zeta\eta$ ratio increases from about 10:1 to 30:1). The levels of both chains begin to increase toward adult levels 2 to 3 weeks after birth. Expression of cell surface TCR changes little over this time period, except that as age advances there is the accumulation of small numbers of single positive, TCR^{bright}, thymocytes.

The susceptibility of immature thymocytes to TCR-mediated deletion was assessed in two ways. First, H57 (a hamster anti-mouse TCR $\alpha\beta$ antibody) or a hamster IgG control was injected into B6 mice on day 19 (day of birth) or day 26 after conception. Thymocytes were collected 48 hrs later and analyzed for viable cell recovery. Inoculation on day 19 reproducibly resulted in about a 50% decrease in viable cell yield, whereas inoculation 7 days later had little effect. Another means of experimentally inducing TCR-mediated thymocyte deletion is to administer "superantigens" that react with all members of a Vβ family. Staphylococcal enterotoxin B (SEB) is recognized by TCRs that contain Vβ3, Vβ7, Vβ8, or Vβ17; exposure of thymocytes to SEB in vivo or in vitro specifically deletes Vβ8$^+$ thymocytes. Therefore, thymic lobes from day 19 or day 26 mice were placed in tissue culture in the presence or absence of SEB. After overnight incubation the expression of Vβ8 or Vβ6 assessed by flow cytometry. SEB caused a substantial decrease (up to 50%) in the number of Vβ8$^+$ thymocytes when added to thymic lobes from day 19 mice. As expected, this was accompanied by a reciprocal increase in the fraction of SEB-resistant Vβ6$^+$ thymocytes. For day 26 the thymic lobes results were quite different: SEB had little effect on the fraction of Vβ8$^+$ cells, and did not cause an increase Vβ6$^+$ cells. To directly test if it was the $\zeta\eta^{hi}$ thymocytes that were being deleted by activation, 4 to 5 wk old B6 mice (with relatively high levels of ζ_2 and $\zeta\eta$) were injected with H57 or hamster IgG. Two days later, thymi were harvested and analyzed by flow cytometry. Anti-TCR treatment caused a 60 to 70% decrease in thymocyte recovery, primarily due to loss of the CD4$^+$CD8$^+$ (double-positive) cells. Thus, thymocytes from day 19 and 4-5 wk old, but not from day 26, mice were susceptible to TCR-mediated deletion, extending the correlation between this phenomenon and $\zeta\eta/\zeta_2$ levels. The ζ and η levels of double-positive thymocytes from H57-treated and untreated mice were analyzed by 2-D nonreducing/reducing SDS-PAGE of radioiodinated total cell membranes after immunoprecipitation with an anti-ζ/η antiserum. The amount of ζ_2 in thymocytes from H57-treated mice was the same as that in cells from hamster IgG-treated mice. Strikingly, η chain expression was clearly less in the H57-treated animals. In fact, the $\zeta\eta$:ζ_2 ratio decreased by 4- to 10-fold after treatment with H57, indicating that TCR-mediated activation of thymocytes from young adult mice preferentially depletes cells that express relatively high levels of $\zeta\eta$. The pattern of susceptibility of thymocytes to activation-induced depletion in early life is remarkably similar to that for neonatal tolerance, in which injection of viable allogeneic cells into mice the day of birth results in long-term tolerance to skin grafts; injection of the same cells several days after birth does not. We speculate that it is the difference in the ζ_2:$\zeta\eta$ ratio that determines the thymocytes' susceptibility to deletion at this stage of life. However, it is also possible that one or more genes co-regulated with ζ and η are responsible for this phenotype. One way to explore the possible mechanism is to determine cell surface levels of TCR-associated ζ_2 and $\zeta\eta$. To this end, we are trying a novel approach that would involve the biotinylation of cell surface proteins, followed by precipitation with insolubilized avidin and immunoblotting for ζ and η. If successful, such an approach would provide a specific and sensitive method to examine the chains that are exclusively expressed on the cell surface.

II. Activation- and Steriod-Induced Cell Death

Glucocorticoids are known to induce apoptosis in T cells. Glucocorticoids bind cytoplasmic receptors that have both ligand- and DNA-binding domains. After binding, the receptor/ligand complex migrates to the cell nucleus where it binds to enhancer sequences (glucocorticoid-responsive elements, or GREs). The pleotropic biological effects of glucocorticoids are mediated by gene products whose transcription is enhanced or inhibited by the receptor/GRE interaction. Because glucocorticoid-induced T cell death has many features in common with that induced by activation, we investigated how these two pathways might interact in murine T-cell hybridomas and clones. Treatment with either dexamethasone (Dex) or specific antigen caused the death of the pigeon cytochrome c-specific murine T-cell hybridoma, 2B4.11. Surprisingly, when 2B4.11 cells were incubated with antigen-presenting cells (APCs) in the presence of Dex plus antigen there was markedly less cell death than when either agent was added alone. The same observation was made when immobilized anti-CD3 antibodies were used to stimulated the cells, eliminating the possibility that the Dex effect was due to its action on APCs. Cyclosporin A (CsA) prevents the activation-induced transcription of numerous lymphokine genes, and blocks the cell death response of T-cell hybridomas and thymocytes in response to activation. Whereas CsA completely prevented 2C11-induced cell death, it did not prevent that caused by Dex. The addition of CsA to the combination of Dex and 2C11 caused a partial reversal of the antagonism, as evidenced by the reappearance of a dose-response relationship with cell lysis as the concentration of Dex was increased. To directly determine if fragmentation of DNA, the hallmark of programmed cell death, was affected by simultaneous treatment with Dex and 2C11, 2B4.11 cells were labelled with [^{3}H]-thymidine and the release of detergent soluble DNA fragments from the nucleus was measured. While Dex or anti-CD3 caused the specific release of substantial amounts of radiolabeled and fragmented DNA, the combination of anti-CD3 and Dex resulted in no specific DNA fragmentation. Thus, as with cell lysis, the DNA fragmentation induced by both glucocorticoids and activation is prevented by concurrent exposure to both stimuli. In addition, CsA completely blocked 2C11-induced DNA fragmentation, but had little effect on Dex-induced DNA fragmentation. Finally, the addition of CsA to 2C11 plus Dex partially reversed the mutual antagonism, just as for ^{51}Cr release.

The kinetics of the antagonism between cellular activation and Dex was investigated by incubating ^{51}Cr-labeled 2B4.11 cells in either 2C11-coated plastic wells or with Dex. At intervals after the initiation of culture, Dex was added to the 2C11-stimulated cells (or 2C11 was used to activate the cells cultured in Dex). Specific release of ^{51}Cr was measured 12 hrs after the cultures were begun. Addition of Dex to 2C11-stimulated cells, or 2C11-stimulation of cells cultured with Dex, at anytime from time 0 (simultaneous addition) up to approximately 2 hrs, prevented virtually all cell lysis. If the second stimulus was added 4 hrs after the first, there was still a 75 to 85% block of cell death. Only when the second stimulus was added 8 hrs after the first was cell lysis determined at 12 hrs maximal. Normal T-cell cones expressed a similar phenotype: activation with antigen or 2C11 prevented the lytic effect of Dex. Furthermore, CsA largely prevented the antagonism, allowing Dex plus 2C11 treated D10 cells to lyse.

The mutual interference of activation and glucocorticoids in the induction of cell death prompted an examination of where the two pathways might intersect. RU-486 is a potent competitive antagonist of glucocorticoids. One μM RU-486 itself had no effect on the viability of 2B4.11 cells. However, it blocked the cytotoxic effects of Dex completely. When similar experiments were performed with 2C11 stimulation, RU-486 had no effect on activation-induced cell death. Another way to examine a potential role for the steroid receptor is to determine how T-cell activation affects the translocation of the steroid receptor from the cytoplasm to the nucleus. To determine the location of steroid receptor, 2B4.11 cells were separated into cytoplasmic and nuclear fractions after activation or exposure to Dex for 1 hr. nuclear proteins were extracted, and both the cytosolic and nuclear proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-glucocorticoid receptor antiserum. One hr after exposure to Dex the cytosolic steroid receptor had decreased by approximately 75%, with the concomitant appearance of the steroid receptor in the nuclear fraction. In contrast, 1 hr after activation with either anti-CD3 or anti-Thy-1 there was little or no decrease in the presence of the steroid receptor in the cytosol of 2B4.11 cells, and no nuclear steroid receptor protein was detected. Importantly, both anti-CD3 and anti-Thy-1 had little effect (inhibitory or enhancing) on the nuclear translocation of the glucocorticoid receptor caused by Dex. To examine these possibilities that activation antagonized the activity of Dex by preventing steroid receptor binding to the GRE, or by preventing effective transcription or translation of GRE-regulated genes, 2B4.11 cells were transfected with PRE-PBL$_7$, a plasmid encoding a thymidine kinase promoter-driven bacterial enzyme, chloramphenicol acetyltransferase (CAT). The amount of CAT gene transcription is regulated by two tandemly placed progesterone/ glucocorticoid-responsive elements (PRE/GRE) 5' of the promoter region. The 2B4.11 cells that were transfected with a construct containing the CAT gene but not the PRE/GRE failed to produce levels of CAT activity above that of mock transfected cells. The 2B4.11 cells transfected with PRE-PBL$_7$ and incubated in medium alone also displayed negligible levels of CAT activity. In contrast, exposure of the PRE-PBL$_7$ transfected cells to Dex caused a substantial increase in CAT enzymatic activity. Stimulation of these transfectants with 2C11 failed to induced CAT activity. Finally, stimulation of the transfectants with 2C11 also failed to prevent the Dex-mediated increase in CAT activity. In fact, the combination of 2C11 and Dex paradoxically induced about two-fold more CAT activity than Dex alone. Together, these data strongly indicate that T-cell activation does not directly utilize the steroid receptor-mediated pathway of programmed cell death. Moreover, the ability of cellular activation to antagonize Dex-induced cell death does not appear to interfere at any point prior to the initiation of GRE-controlled gene transcription.

III. Physical Associations Between Biologically Active T-Cell Molecules

In addition to the TCR, a number of plasma membrane molecules of unknown function can initiate signals leading to cellular activation events indistinguishable from those caused by TCR occupancy. Among the best characterized of these molecules is Thy-1. A pertinent observation is that Thy-1 is not normally found as a transmembrane molecule, but is anchored to plasma membrane lipids via a glycosyl-phosphatidylinositol linkage. Therefore, the transfer of information from the outside of the cell to the inside most likely involves the participation of

additional signal transducing elements. We have proposed that Thy-1 mediates it biological functions by interacting with one or more transmembrane molecules. Studies using homobifunctional chemical cross-linkers have allowed us to demonstrate specific, intimate, contact between Thy-1 and CD45, a transmembrane tyrosine phosphatase. Moreover, the TCR was also found to co-precipitate with CD45 from lysates of chemically cross-linked T-cell membranes. Quantitation of the association of Thy-1 and the TCR with CD45 was performed in two ways. First, the tyrosine phosphatase activity of chemical complexes was measured in immunoprecipitates of Thy-1 or the TCR. Using the best estimates of cell surface molecule number, it appears that 10 to 20% of Thy-1, and 35 to 80% of the TCR, can be cross-linked to CD45. Another approach was to immunoprecipitate CD45 from chemically cross-linked T-cell lysates and immunoblot the material with an anti-TCR ζ antiserum. With this method it was estimated that between 19 and 37% of cell surface TCRs were associated with CD45. Finally, the association between these molecules could be observed in the absence of cross-linking when digitonin, a relatively nondisruptive detergent, was used to prepare the lysates. Together, these results support a significant physical interaction between these biologically active molecules.

To determine if there CD45 played a functional role in TCR or Thy-1 mediated cellular activation, we have attempted to make T-cell mutants that lack CD45. Such mutants have been made in collaboration with Francis Dumont, who used ethyl methanesulfonate and repetitive cell sorting to obtain $CD45^-$ variants of the YAC-1 murine T-cell line. The mutants express low to undetectable levels of CD45 mRNA and express no detectable CD45 on the cell surface. All of the cells are TCR^+ and $Thy\text{-}1^+$. Preliminary experiments have revealed that in response to TCR perturbation by antibody, the YAC-1 wild type cells mobilize intracellular Ca^{2+}, turn over phosphoinositides, and secrete small amounts of a lymphokine that supports the growth of HT-2 cells (probably either IL-2 or IL-4). Interestingly, the $CD45^-$ mutants do none of these things. To determine if it is truly the loss of CD45 that results in this phenotype, we have obtained the T- and B-cell isoforms of the CD45 cDNA and have cloned them into eukaryotic expression vectors. The T cell form has not been transfected into one of the $CD45^-$ YAC-1 mutants, and $CD45^+$ isolates are now being expanded. We will shortly test the hypothesis that CD45 expression is necessary for transmembrane signal transduction and biological activity of Thy-1 and the TCR.

SIGNIFICANCE

Elucidation of the mechanisms that determine whether a T cell will proliferate or die after activation is a prerequisite for any detailed understanding of the phenomenon of negative selection. The clues obtained from the 2B4.11 T-cell hybridoma system have suggested the TCR composition may have a critical influence in this regard, and the recent observations that the expression of the TCR $\zeta\eta$ heterodimer in immature thymocytes is highly regulated lends credence link expression of $\zeta\eta$ with susceptibility to TCR-mediated deletion. The simplest interpretation of the results is that $\zeta\eta$ TCRs are specifically responsible for coupling TCR occupancy to programmed cell death. It is also possible that one or more genes co-regulated with $\zeta\eta$ subserve this function. In any case, these observations provide the first insights into possible mechanisms underlying thymocyte signalling to die, and provide the basis for future experimentation.

In an analogous fashion, the observation that glucocorticoids and cellular activation are mutually antagonistic adds a new "rule" to the phenomenon of programmed cell death. Extension of this finding to normal cells, in vitro or in vivo, should allow an increase in our understanding of both the mechanisms and physiological role of activation-induced programmed cell death.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09310-04 OAD

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BTS, NCI
Others:	J. Zuniga-Pflucker	General Fellowship Program	OAD, BTS, NCI
	J. Fine	Fellow	OAD, BTS, NCI
	D. D. Rowe	Biologist	OAD, BTS, NCI

COOPERATING UNITS *(if any)*

EIB, NCI (D. Singer); LEI, BRMP, NCI-FCRDC (H. Young)

LAB/BRANCH

Office of the Associate Director

SECTION

Biological Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

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: 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: blocking of the IL-2R results in arrested T-cell development also in this model. Also, the functionality of IL-2R was determined by Scatchard-plot analysis; the affinities of IL-2R on developing thymocytes (a high-affinity and low-affinity) were remarkably similar to those on IL 2-dependent cell lines. Finally, a panel of early fetal thymic cell lines has been derived by v-myc/v-raf tranformation, and is currently being analyzed with respect to the status of expression of all T cell differentiation antigens, and susceptibility of several differentiation inducing regimens.

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

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD, BTS, NCI
Juan Zuniga-Pflucker	General Fellowship Program	OAD, BTS, NCI
Jay Fine	Fellow	OAD, BTS, NCI
Dorothy D. Rowe	Biologist	OAD, BTS, 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 of 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 thyocytes 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 $\gamma\delta$ or non-receptor bearing cells in development, i.e., production of lymphokines. Current studies focus on which other cell surface molecules are involved in the delivery of successful activation signals to early T cells. Also, an antisense IL-2 construct has, upon in vitro transfection of IL-2 producing cell lines, been demonstrated to block IL-2 production completely. This construct will now be used to produce transgenic mice with a constitutively inability to produce IL-2. Finally, the regulation of T-cell differentiation is studied with a panel of v-myc/v-raf transformed cell lines representative of different stages of fetal thymic development.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09311-04 OAD

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BTS, NCI
Others:	B. Niklinska	Visiting Fellow	OAD, BTS, NCI
	S. Wu	Howard Hughes Scholar	OAD, BTS, NCI

COOPERATING UNITS *(if any)*
LCBM, NICHHD, Bethesda, MD (T. Rutlege, S. Frank, R. Klausner); EIS, NCI, Bethesda, MD (D. Segal)

LAB/BRANCH
Office of the Associate Director

SECTION
Biological Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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.)*

The TCR ζ chain, either in the native or a mutated form, has been transfected into ζ chain-negative variants of the murine T-cell hybridoma 2B4.11. Six different ζ species have been studied in detail. All forms of ζ restored cell surface TCR levels to normal levels. The full length ζ transfectant (FL) was able to respond to antigen or anti-CD3 antibodies by producing IL-2 and being growth inhibited. A transfectant in which ζ was truncated at residue 150 (clipping off the last 14 amino acids of the chain) responded well to anti-CD3, but poorly to antigen. Another transfectant in which ζ was truncated at residue 108 responded only to anti-CD3; no IL-2 or growth inhibition was found when antigen was used as the stimulus. Two point mutations resulted in the same phenotype. GV135 is a cell line in which a Gly->Val substitution was made at residue 135; YT153 is a transfectant in which a Tyr->Thr substitution was made at residue 153. These substitution would be predicted to alter the consensus nucleotide-binding site and the ultimate phosho-tyrosine, respectively. In contrast, a substitution of a Lys for an Arg at residue 150 resulted in a transfectant that was indistinguishable from the wild type ζ chain in its biological responses to activation. These data indicate that the ζ chain plays a crucial role in transmembrane transduction of activating signals. Furthermore, they indicate that occupancy of the TCR with ligand is qualitatively dissimilar from cross-linking with multivalent reagents. The difference between antibody and antigen could not simply be due to the different TCR chains involved, since antibodies directed to TCR α or TCR αβ were identical to anti-CD3 antibodies in this regard. Finally, in contrast to ζ, transfection of δ chains that had the entire intracytoplasmic portion truncated yielded cells that responded as well as cells transfected with the full length δ chain. These data emphasize the moldular nature of the TCR complex, and strengthen the model that different chains or sets of chains subserve specific and discrete functions.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Investigator	OAD, BTS, NCI
Barbara Niklinska	Visiting Fellow	OAD, BTS, NCI
Samuel Wu	Howard Hughes Scholar	OAD, BTS, NCI

OBJECTIVES

(1) To analyze the structural and functional roles of the different components of the T-cell 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. Mutational Analysis of the TCR Invariant Subunits

In collaboration with Stuart Frank and Richard Klausner we have studied the biological properties of TCRs that contain mutated ζ chains. Five different forms of ζ chain have been transfected into ζ chain-negative 2B4.11 variant: full length (FL), truncated at amino acid 108 (CT 108) or 150 (CT 150), a single amino acid substitution at residue 135 (Gly->Val; GV135), or a single amino acid substitution at residue 153 (Tyr->Thr; YT153). In all cases, cell surface TCR expression was restored toward normal (although the TCR on the CT150 transfectant was only about 50% that of the wild type cell). Activation-induced IL-2 production in these clones was assessed. Stimulation of FL, CT108, and CT150 cells with antigen revealed a clear hierarchy. FL cells responded well, generating detectable IL-2 when stimulated with as little as 1 μM pigeon cytochrome c fragment 81-104 (fragment 81-104). CT150 cells responded poorly, yielding only low levels of IL-2 at relatively high antigen concentrations (10 to 30 μM). CT108 cells consistently produced no IL-2 at antigen concentrations up to 30 μM. Stimulation with an immobilized anti-CD3 mAb, 2C11, gave a somewhat different result. FL cells still produced the most IL-2, with plateau levels within two-fold of that induced by antigen. In contrast to antigen, when stimulated with 2C11 both CT150 and CT108 cells produced easily detectable IL-2, although still substantially less than FL cells (maximal levels produced by CT150 and CT108 being generally 15 to 20% of that produced by FL). Furthermore, whereas CT08 never produced IL-2 when stimulated with antigen, it was at least as good, if not better, than CT150 when responding to anti-CD3 mAbs. The immobilized anti-α mAb, A2B4-2, and the anti-$\alpha\beta$ antibody H57, stimulated a pattern of IL-2 production indistinguishable from 2C11. G7, a mitogenic anti-Thy-1 mAb, stimulates IL-2 production from T cells. In contrast to the anti-TCR antibodies, G7 caused only the FL cells to produce IL-2. The inhibition of transformed growth that follows activation is another quantitative measure of T-cell activation. As with IL-2 production, antigen caused the most growth

inhibition in FL cells. CT150 cells were slightly inhibited, and only at high concentrations of antigen, whereas CT108 cells were not inhibited at all. Stimulation with immobilized anti-TCR antibodies caused all cells to slow their growth equally.

The C-terminal tail of ζ contains a consensus nucleotide binding site, Gly-X-Gly-X-X-Gly similar to that found in tyrosine and serine/threonine kinases. In the GV135 transfectant this sequence is disrupted in a way that would prevent nucleotide binding in these enzymes. This mutant gave pattern of IL-2 production in response to antigen, G7, and anti-TCR antibodies that was similar to CT108 cells. Furthermore, this same pattern was demonstrated in YT153 cells, mutants in which the C-terminal-most tyrosine was eliminated. Interestingly, in this last construct occupancy of the TCR does not lead to tyrosine phosphorylation of the ζ chain, despite the fact that 5 other tyrosine residues remain as potential phosphorylation sites. Removal of this single tyrosine may prevent the activation of a tyrosine kinase. Alternatively, it may prevent phosphorylation only of ζ, either because it acts cooperatively with the other tyrosine residues and the kinase, or because its presence somehow retards dephosphorylation by tyrosine phosphatases such as CD45. In any case, it is apparent from these data that the functional effects of deleting portions of the cytoplasmic tail of ζ differ depending upon both the stimulus and the nature of the mutation. These results are the first to directly demonstrate the importance of the intracellular ζ chain in coupling the cell surface TCR to more distal signalling events.

In addition to ζ deficient mutants, we also have obtained mutants of 2B4.11 cells that are 98% deficient in expression of CD3-δ. These cells have been transfected with either full length δ chain or with δ chains which have been truncated just after the transmembrane portions (that is, they are inserted through the plasma membrane and have a normal extracellular portion, but lack an intracellular piece). Cells transfected with the mutant δ chains responded to antigen, anti-TCR antibodies, and anti-Thy-1 much the same as did cells transfected with the full length δ chain. As far as can be deducted from these studies, and in marked contrast to the ζ mutants, intracellular CD3-δ is not necessary for normal TCR function.

We conclude from the studies of ζ chain mutants that signalling initiated by TCR occupancy is qualitatively dissimilar from signalling initiated by TCR cross-linking. The physiological basis for this distinction is unclear. The differences between the two modes of stimulation that might matter in this regard include: conformational changes (antigen) vs. cross-linking (antibody), low affinity (antigen) vs. high affinity (antibody), monovalent (antigen) vs multivalent (antibody), membrane anchored (antigen) vs. immobilized (antibody), presence of accessory molecules (antigen) or not (antibody). To approach this question, heterochimeric antibodies that are designed to "mimic" antigen are being created. Anti-TCR α Fab fragments are chemically cross-linked to anti-MHC class II Fab fragments. Such antibodies should be monovalent for both the TCR and class II molecules and bind the two molecules that are normally involved in antigen presentation. Preliminary results have established that these antibodies stimulate IL-2 production from 2B4.11 cells, and that this requires the presence of accessory cells bearing the appropriate MHC class II molecules. Soluble antibodies to either the TCR or class II molecules block the stimulatory action

of the heteroconjugates. We will be able to discriminate between some of the models that account for the difference between antigen and anti-TCR antibodies by using these heteroconjugates to stimulate the mutant ζ chain transfectants.

SIGNIFICANCE

How T cells transduce signals across the plasma membrane is one of the central issues of modern immunology. Its complex, multi-subunit, structure has led us to suggest that different sets of chains have different functional properties. The availability of T-cell mutants that lack one or more of these chains, and clones of the genes that encode these chains, has allowed us to reconstruct a cell surface TCR that expresses well defined alterations. These studies have shown that the ζ chain, which has a longer intracytoplasmic portion than any of the CD3 subunits, is critical in allowing TCR occupancy to result in IL-2 secretion. Furthermore, disruption of the nucleotide concensus binding region or the terminal tyrosine residue appear to cause as much of an effect as truncation of large regions of the intracellular portion. Interestingly, this function appears to be bypassed when TCR cross-linking with antibody is used to stimulate the cells. In contrast to ζ, the intracytoplasmic portion of CD3-δ does not appear to be necessary for coupling TCR occupancy to Il-2 production. Manipulation of the genes is a powerful approach that will allow a detailed understanding of TCR structure-function relationships. Such studies will ultimately allow one to map the chains and regions of this receptor that contribute to its critical signalling functions.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09323-03 OAD

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BTS, NCI
Others:	L. Jones	Fellow	OAD, BTS, NCI
	J. Zuniga-Pflucker	General Fellowship Program	OAD, BTS, NCI
	L. T. Chin	Howard Hughes Fellow	OAD, BTS, NCI

COOPERATING UNITS *(if any)*

Experimental Immunology Branch, NCI (A. Singer)

LAB/BRANCH

Office of the Associate Director

SECTION

Biological Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

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 V-regions of particular TCR-chains 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 generation of 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 significance 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.

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD, BTS, NCI
Lori Jones	Fellow	OAD, BTS, NCI
Juan Zuniga-Pflucker	General Fellowship Program	OAD, BTS, NCI
L. Thomas Chin	Howard Hughes Fellow	OAD, BTS, NCI

OBJECTIVES

To determine the role of CD4, CD8, T-cell receptor (TCR) and major histocompatibility complex (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. In addition, a signalling role for CD4 and CD8 in immature thymocytes was established. Finally, the phenomenon of clonal deletion was found to be exquisitely dependent on the thymus, since this mechanism of tolerance acquisition was absent in nude mice and in thymectomized mice.

PUBLICATIONS

Veillette A, Zuniga-Pflucker JC, Bolen JB, Kruisbeek AM. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways, J Exp Med 1989;170:1671-80.

Fry AM, Jones LA, Kruisbeek AM, Matis LA. Extrathymic T cell maturation represents a distinct developmental pathway, Science 1989;246:1044-6.

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SUMMARY REPORT

BIOLOGICAL RESOURCES BRANCH

October 1, 1989 through September 30, 1990

INTRODUCTION

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

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 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	Paul J. Miller, M.S.
Secretary	Virginia L. Axline
Clerk-Typist	Dawn M. Crigger
Editorial Assistant	Nancy Parkhurst

PRECLINICAL STUDIES GROUP

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.
Jon T. Holmlund, M.D.

BIOLOGICAL RESOURCES BRANCH, PRECLINICAL STUDIES GROUP

This group is responsible for monitoring the results of Biological Response Modifiers Program-supported preclinical research as well as testing, production, and development contracts supported by the Biological Response Modifiers Program. 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 Biological Response Modifiers Program 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 BRM Operating Committee (BRMOC), Biological Response Modifiers Program-Cancer Therapy Evaluation Program Working Group (BCWG), the Biological Response Modifiers Program-Developmental Therapeutics Program Working Group (BDWG), and the combined Biological Response Modifiers Program-Cancer Therapy Evaluation Program-Developmental Therapeutics Program Working Group (BCDWG) for the development of biologicals.

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 of the Division of Cancer Treatment, to coordinate appropriate development of investigational agents for clinical trials, and for regulatory issues.

DIVISION OF CANCER TREATMENT BIOLOGICAL RESPONSE MODIFIERS OPERATING COMMITTEE

Coordination of the clinical development of BRMs through preclinical studies, production, and pilot clinical trials is done jointly with the Cancer Therapy Evaluation Program and Developmental Therapeutics Program personnel through the BRM Operating Committee, formerly known as the BCWG. This group assembles and presents data and recommendations on BRMs to the Division of Cancer Treatment-Decision Network Committee (DCT-DNC), and performs staff work to implement DNC decisions. This Group also coordinates Division of Cancer Treatment efforts in the planning and review of Phase III clinical trials which include BRMs.

BIOLOGICAL RESPONSE MODIFIERS OPERATING COMMITTEE (BRMOC)

The BRMOC acts as the steering committee for the BRMP extramural program. The BRMOC reviews and prioritizes agents as to evaluation in the preclinical screens. The recommendation of the BRMOC are subsequently presented to the DNC and in turn the recommendations of the DNC are implemented by the BRMOC and program staff. Members of the BRMOC 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.

SUMMARY OF FY 90 ACTIVITIES

During this year the BRB has been involved in the following:

* Maintained 26 contracts for testing BRMs in Phase I clinical trials.

* Maintained three 5-year contracts for Phase I clinical trials of BRMs, "Task A monoclonal antibodies and other targeting molecules."

* Maintained four additional 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 National Institutes of Health-supported investigations.

* Chelated three different 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-2, IL-3, IL-4, IL-6, tumor necrosis factor (TNF)-alpha, granulocyte/macrophage-colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (M-CSF) and transforming growth factor (TGF)-beta.

* During FY 90, the BRB awarded $5.3 million for extramural contracts, including $2.8 million in clinical contracts and $2.5 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 reviews.

Liaisons have been established and maintained with other programs in the National Cancer Institute, including the Immunology and Tumor Biology Programs in the Division of Cancer Biology Diagnosis and Centers, to minimize overlap in the grant and contract areas. A regular working relationship is maintained with the Developmental Therapeutics Program of the Division of Cancer Treatment to coordinate drug and biologic development. A cooperative clinical protocol evaluation system with the Cancer Therapy Evaluation Program, Division of Cancer Treatment has been established to coordinate Phase I, Phase II, and Phase III BRM clinical trials.

ACCOMPLISHMENTS OF THE BRMP GRANT PROGRAM IN FY 90

A total of 103 grants were supported by the BRMP with $28.9 million during the period October 1, 1989 through June 5, 1990. There were 72 Research Projects (R01), 10 Program Project (P01), 1 Outstanding Investigator Grant (R35), 2 Phase I SBIR (R43), 2 Phase II SBIR (R44), 8 Merit Awards (R37), 1 Conference Grant (R13), 6 First Investigator Research Grant (R29) (Tables I & II).

Approximately one-third of current Biological Response Modifiers Program-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 Biological Response Modifiers Program-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 mouse. 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 I-131-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 Cu-67 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. 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 Biological Response Modifiers Program 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.

New Grants

Grant projects funded for the first time in FY 90 included the isolation, characterization and expansion of tumor-specific effector cells, novel human monoclonal antibodies for leukemia treatment, the antitumor effects of anti-idiotypic antibodies, the use of combinations of cytokines in differentiation therapy which reduces tumor-specific immune suppression, and the enhancement of tumor immunity by genetic manipulation of histocompatibility antigens.

Request for Applications

In FY 90, three awards were issued for the FY 89 RFA, "Studies of Chronobiological Effects in Cancer Treatment with Biological Response Modifiers and/or Drugs." One application to a previously issued RFA (FY 87), "Manipulation of the Suppressor Arm of the Immune Response Directed Towards Successful Human Immunotherapy" will be funded this year as a result of an amended 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 90, the Biological Response Modifiers Program and the Developmental Therapeutics Program issued one Program Announcement: Specific Cancer Cell Targeting Using Molecular Genetic Technology. In FY 90, there have been 7 applications received in response to Biological Response Modifiers Program

Program Announcements. One has been funded on the topic of Specific Cancer Cell Targeting.

BRMP CONTRACT PROGRAM

The BRB received five responses to the Request for Procurement (RFP) entitled: "Production of Antisense Oligonucleotides for In Vitro Use and Animal Studies." The Source Evaluation Group selected four (4) offerors to receive a Master Agreement. Four Task Orders were competed during this fiscal year, and Synthecell Corporation of Rockville, Maryland was selected to perform these Master Agreement Orders. Due to the departure of several National Institutes of Health investigators, only two Task Orders were awarded. Two additional Master Agreement Orders are anticipated to be awarded in late FY 90 or early FY 91.

A new RFP entitled: "Preparation of Monoclonal Antibody Chelates for use in Radioimmunotherapy" is scheduled for review in the summer of 1990. This Master Agreement mechanism should be awarded in late FY 90 or early FY 91. A specific task order has been included in this RFP for the chelation of the monoclonal antibody, 14G2a, under Good Manufacturing Practices conditions.

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 14 extramural institutions for testing BRMs in Phase I clinical trials (Table IV).

The BRB is in its third and final year of a contract with Abbott Biotech (formerly Damon Biotech) for the production of large-scale clinical-grade monoclonal antibodies. Greater than 200 grams of material have been delivered to the BRB.

In FY 90, the BRB received 100 grams of a total of 150 grams of monoclonal antibody R24 from Celltech Limited, which is a subcontract of Program Resources, Incorporated. A contract through Program Resources, Incorporated was finalized with Brunswick Biotechnetics for the production of clinical-grade anti-GD2 monoclonal antibodies.

The BRB continued the distribution of IL-2 reference standard to the scientific community through the expanded function of the Biological Response Modifiers Program 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 in FY 90 to nearly 2,000. Additionally, the repository was expanded to include international standards from the National Biological Standards Board Repository in England.

During this fiscal year, the Branch issued a Small Business Innovative Research (SBIR) RFP for one Phase I and one Phase II SBIR contract. Proposals from these RFPs are still in review.

BRB staff reviewed and visited all required preclinical contracts during the fiscal year.

The BRB continued its collaboration with the Cancer Therapy Evaluation Program in the BCWG, which serves an expanding role coordinating the BRM drug development programs of the Biological Response Modifiers Program and the Cancer Therapy Evaluation Program. Representatives of the National Institute of Allergy and Infectious Diseases also participated when Acquired Immune Deficiency Syndrome (AIDS)-related issues were discussed.

The BRB/Biological Response Modifiers Program is now working with a new group composed of representatives from the Cancer Therapy Evaluation Program and the Developmental Therapeutics Program for the purpose of planning and obtaining resources for the development of biologicals for clinical trials. This new working group, the BRMOC, will review promising new biologicals for presentation to the DNC as well as develop plans for the production and preclinical/clinical evaluation of those biologicals which are approved by the DNC.

CONFERENCES AND MEETINGS

* Two members of the BRB were on the organizing committee which resulted in a Division of Cancer Treatment-sponsored workshop in Baltimore, Maryland entitled: "Combining Biological Response Modifiers With Cytotoxics in the Treatment of Cancer: Developing a Rational Approach to a New Therapy." This workshop was held on March 5-7, 1990, and approximately 30 speakers discussed the current state of knowledge with respect to integrating various BRMs into chemotherapeutic regimens. The major sessions included the broad topics of: "Enhancement of BRMP Antitumor Activity by Cytotoxic Agents; The Enhancement of Cytotoxic Antitumor Activity by BRMs; Preclinical Evaluations and Clinical Correlations of Drug/BRM Interactions; and Regulatory Considerations in the Development of Drug/BRM Combinations." A manuscript describing the proceedings of the meeting is in preparation for publication in the Journal of the National Cancer Institute.

* On June 11-12, 1990, the BRB co-sponsored with the Division of Cancer Biology Diagnosis and Centers, Cancer Therapy Evaluation Program and Janssen Research Foundation a "Workshop on Levamisole: Mechanisms of Antitumor Activity."

* Therapeutic Application Group meetings on the following topics: preclinical development of IL-8; therapeutic use of IL-1, results of Phase I clinical trials; clinical development of IL-4; interferon-gamma in solid tumor patients; and preclinical development of IL-3.

* The BRB also sponsored the Biological Response Modifiers Program Friday Seminar Series, which brought 45 leading scientists to Frederick to interact with the Biological Response Modifiers Program staff and to present a seminar on their work.

PHARMACEUTICAL COMPANY RELATIONSHIPS

The Biological Response Modifiers Program has established relationships with most of the biotechnology and pharmaceutical companies which produce BRMs. Relationships exist with Ajinomoto, Amgen, Becton Dickenson, Biogen, Brunswick,

Burroughs-Wellcome, Ciba-Geigy, Celltech, Cetus-Shell, Collaborative Research, Dainippon, Genentech, Genetics Institute, Hoechst-Roussel, Hoffmann-LaRoche, Hybritech, Immunex, Janssen, Lederle (American Cyanamid), Ortho, Rorer, Sandoz Research Institute, Schering, Seragen, Sterling (Eastman-Kodak), 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 90.

* 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 90.

* Establishment of a new Master Agreement mechanism for clinical trials involving complex or sophisticated immunological manipulations for the treatment of cancer or AIDS.

* RFP announcements, competition and source selection for recompetition of the BRM Repository contract (currently held by Hazleton Biologicals).

PUBLICATIONS

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PUBLICATIONS (Continued)

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TABLE I

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

GRANT EXPENDITURES FOR FY 90 THROUGH JUNE 1990

TYPE OF GRANT	NUMBER	TOTAL COST AWARDED
Research Projects (R01)	72	$12,559,685*
Program Projects (P01)	10	12,413,116
Small Business Innovative Research (SBIR)	4	521,503
Conference Grants (R13)	1	15,000
Merit Awards (R37)	8	2,122,690
First Awards (R29)	6	603,673
Outstanding Investigator Award (R35)	1	678,810
TOTALS	102	$28,914,477*

*Included in this category are 7 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"
CA28149	Vitetta, E. S.	"New Strategies for Immunotoxin Therapy"
CA47334	Epstein, A. L.	"Immunotherapy of Human Lymphomas With Lym-1 and Lym-2"
CA50054	Posner, M. R.	"Human MoAbs to Study and Treat Leukemias"
CA47860	Bhattacharya-Chatterjee, M.	"Idiotype Approach to Therapy of Human T-Cell Leukemia"
CA48713	Samoszuk, M. K.	"Enzyme Immunoaugmentation of Hodgkins Disease"
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"

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 and Lymphoma - Clinical (Continued)		
CA37497	Royston, I.	"Monoclonal Antibodies in Cancer Detection and Treatment"
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"
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"
CA43904	Shively, J. E.	"Colon Cancer and Engineered Antibodies"
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"

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 - Clinical (Continued)		
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"
CA50633	Weiner, L. M.	"Bispecific Anti-Tumor FCYRIII Monoclonal Antibodies"
Monoclonal Antibody Localization		
CA43544	Murray, J. L.	"Optimization of Monoclonal Antibody Localization"
Multi-Drug Resistance - Preclinical		
CA48146	Hochman, J. D.	"Monoclonal Antibodies to Human Multi Drug Resistance"
CYCLOPHOSPHAMIDE		
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity By Cyclophosphamide"
Preclinical		
CA30088	Dray, S.	"Synergy of Tumor Chemotherapy and Host Immunity"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
MOLECULAR BIOLOGY		
Preclinical		
CA48162	Croop, J. M.	"Immunologic Analysis... Multidrug Resistant Gene Family"
CA45667	Geahlen, R.	"Inhibitors Protein Myristoylation as Anti-Cancer Drugs"
CA41746	Murphy, J. R.	"Diptheria Toxin-Related Peptide Hormone Gene Fusions"
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"
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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
TUMOR VACCINES		
Preclinical		
CA29592	Kahan, B. D.	"Active Specific Immunotherapy in Man: A murine model"
CA51434	Kohler, H.	"Preclinical Anti-Idiotype Therapy of Cancer"
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity by Cyclophosphamide"
CA36233	Mitchell, M. S.	"Specific Active Immunotherapy of Human Melanoma"
LYMPHOKINES OTHER THAN IL-2		
Interferons - Preclinical		
CA44262	Esteban, M.	"Mechanisms of Action of Interferon"
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"
Interleukin-1 - Preclinical		
CA38043	Lachman, L. B.	"Biologic Studies of Human Interleukin-1"
CA45143	Gaffney, E. V.	"Malignant Cell Growth Modulation by Interleukin-1"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LYMPHOKINES OTHER THAN IL-2		
Interleukin-1 - Preclinical (Continued)		
CA46259	Gallicchio, V. S.	"Control of Hematopoietic Toxicity With Interleukin-1"
CA48077	Johnson, C. S.	"Cytokine Enhancement of Chemotherapeutic Efficacy"
CA49143	Brunschweiger, P. G.	"Antitumor Activity of Recombinant Interleukin 1"
Tumor Necrosis Factor - Preclinical		
CA44365	Sehgal, P. B.	"Tumor Necrosis Factor Induces a New Regulatory Cytokine"
CA50780	Economou, J. S.	"Therapeutic Regulation of TNF in IL-2 Immunotherapy"
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"
CA35845	Sidman, C. L.	"Purification and Immunobiology of B-cell Maturation Factors"
Other Lymphokines - Preclinical		
CA45672	Lilly, M.	"Therapeutic Uses for Human Granulocyte CSF"
LAK +/- IL-2		
Preclinical		
CA47097	Ochoa, A. C.	"Long-term LAK Cells: Effectors and In Vivo Relevance"
CA45484	Eberlein, T. J.	"Alternative Method of Adoptive Immunotherapy"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LAK +/- IL-2		
Clinical		
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"
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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
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"
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		
CA48172	Kovacs, C. J.	"Cytokine Sequencing and Toxicity: Marrow and GI Studies"
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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
METASTASIS		
Preclinical		
CA30276	Niederkorn, J. Y.	"Immunologic Modulation of Ocular Tumor Metastasis"
CA49950	Barna, B. P.	"Synthetic CRP Peptide--Therapy for Cancer Metastases"
TOXINS		
Preclinical		
CA42354	Maxwell, I. H.	"Tumor Cell Killing by Expression of a Toxin Gene"
CA41746	Murphy, J. R.	"Diptheria Toxin Related Peptide Hormone Gene Fusions"
SBIR		
Phase I		
CA50836	Sullivan, S. M.	"Immunoliposome Delivery of Chemo-therapeutics"
CA53006	Wright, S. C.	"Purification and Characterization of of a TNF Inhibitor"
Phase II		
CA41846	Miller, R. A.	"Shared Tumor Idiotypes of B-Cell Neoplasms"
CA43405	Rhodes, B. A.	"Radiolabeled Antibody Delivery System Quality Control"
OTHER TOPICS		
Preclinical		
CA24628	Nowotny, A. H.	"Relation of Structure to Function in Endotoxin (ET)"
CA47324	Cox, D. C.	"Murine Tumor Immunotherapy Using Non-infectious Reovirus"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
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"
CONFERENCE		
CA52034	Royston, I.	"5th International Conference on Monoclonal Immunoconjugates"
Chronobiology - Preclinical		
CA50749	Hrushesky, W. J.	"Chronobiological Investigation of TNF and IL-2"
CA50752	Fleischman, W. R.	"Chronobiology of Interferon Therapy"
CA50892	Dye, E. S.	"Influence of Bioperiodicity on Tumor Immunotherapy"

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 90

CONTRACTS	Estimates ($K)
Preclinical	
Chemical Coupling of Cytotoxic Agents to Tumor Reactive Monoclonal Antibodies	$ 100
Collection, Storage, Distribution, and Quality Assurance of BRM	450
Animal Lymphokine Evaluation	217
Preclinical Evaluation Laboratory	623
Animal Production	300
Specialized Antibody Production	13
Monoclonal Antibody Purchases	440
Oligonucleotides	316
Clinical	
Master Agreement Orders	510
Phase I Contracts	
Task A: Phase I Clinical Trials of Monoclonal Antibodies	1,043
Task B: Phase I Clinical Trials of Cytokines	1,270
Total	$5,282

SUMMARY REPORT

LABORATORY OF MOLECULAR IMMUNOREGULATION

October 1, 1989 through September 30, 1990

INTRODUCTION

Headed by Dr. Joost J. Oppenheim, the Laboratory of Molecular Immunoregulation (LMI) performs basic research studies on host defense mechanisms. Cytokines are the primary focus of the LMI, and these defense molecules are studied at a number of levels: discovery and development of new cytokines, regulation of cytokine production at the gene level (promoters and transcription factors), the action of cytokines on target cells (studies of receptors, second and third messengers), and cytokine regulation of physiological processes (inflammation, immunity and hematopoiesis). The LMI consists of three sections: Lymphokine, Immunobiology and Cytokine Molecular Mechanisms. In addition, a fourth research group in the LMI concentrates on studies of inflammatory cytokines, operates through the Office of the Chief.

RESEARCH MANAGED THROUGH THE OFFICE OF THE CHIEF

Three LMI investigators are managed through the office of the chief. Drs. Oppenheim and Kouji Matsushima are working on cytokines involved in hematopoiesis and inflammation, and Dr. Scott Durum is studying cytokines involved in immunity.

Dr. Oppenheim, the laboratory chief, previously observed that IL1 is radioprotective in mice, and that the effect was due to radioprotection of hematopoiesis. These studies were performed in collaboration with Dr. R. Neta of the Armed Forces Radiobiology Research Institute (AFRRI). They have extended these studies to understand how IL1 protects hematopoiesis from irradiation. They found that IL1, given to lethally irradiated mice together with bone marrow cells, strongly promoted the ability of small numbers of bone marrow cells to reconstitute the mice. Thus, at least one aspect of the IL1 radioprotective mechanism is to promote hematopoietic reconstitution from stem cells that would be too few in number to accomplish this reconstitution without IL1. Both syngeneic and allogeneic bone marrow grafts were promoted by IL1. Long-term survivors of allogeneic grafts were tolerant to both host and donor histocompatibility antigens and were immunologically competent. These findings have promising clinical applications for patients undergoing bone marrow transplantation for a variety of neoplastic and hematopoietic disorders.

Dr. Oppenheim has also studied the ability of cytokines to retard or promote the transformation of cells from a pre-cancer cell type to a fully transformed cancer cell type. These studies were performed in collaboration with Drs. N. Colburn (CBS, LVC, NCI), F. DeBenedetti and C. Faltynek (LPB, NCI). These studies involved JB6, a nontumorigenic murine epidermal cell line. In response to tumor promoters, such as PMA, JB6 undergoes irreversible phenotypic modification, becoming anchorage independent, as measured by colony formation in soft agar, and it becomes

tumorigenic in nude mice. This transformation process was known to be inhibited by anti-promoters, such as retinoids. Dr. Oppenheim has now found that the cytokine TGFα inhibits the ability of PMA to transform JB6 cells. This TGFα anti-promoter mechanism is probably different from the retinoid mechanism, since the two agents have synergistic effects. These findings suggest that some neoplasias may develop because TGFα is absent from their milieu, or that they have lost the ability to respond to TGFα. This also suggests that TGFα offers a potential therapy for pre-cancer states. On the other hand another cytokine, TNFα, was found to have the opposite effect of TGFα. TNFα promoted the transformation process and synergized with PMA, suggesting a potential role for TNFα in carcinogenesis.

Dr. Matsushima, at present a Visiting Scientist, has studied the signals delivered by IL1 to a cell. He and his colleagues have identified an early intracellular event: the activation of a serine kinase that is distinct from others described previously (PKC, PKA and casein kinase). They have cloned the major cellular substrate of this serine kinase and identified it as 1-plasmin. Several other cellular stimuli, including TNFα and PMA, which share many activities with IL1, also appear to use this kinase-substrate pathway. These cytosolic events induced by IL1 are followed by nuclear events. To understand these nuclear events, they have examined a promoter element on the IL8 gene in an effort to determine how this element responds to IL1. They have localized the IL1-responsive region, are characterizing the proteins that bind to this region, and examining whether the serine kinase they previously identified in cytosol may activate the DNA binding proteins by phosphorylating them. These studies could lead to the first fully characterized biochemical pathway leading from a cytokine receptor to the activation of a gene.

Dr. Matsushima and colleagues previously cloned and expressed two cytokines and are studying various aspects of these cytokines. IL8 is a chemotactic and activating factor for neutrophils. The protein structure of IL8 has been revealed by nuclear magnetic response and x-ray crystallography. The biological activities of IL8 have been studied in vivo, and shown to cause accumulation of neutrophils and lymphocytes at sites of administration. The cellular receptors for IL8 on neutrophils were characterized. MCAF is a chemotactic and activating factor for monocytes. They have shown that, in vivo, MCAF causes accumulation of monocytes at sites of administration. They have identified specific receptors for MCAF on monocytes, and identified a number of responses of monocytes to MCAF, including increased tumor killing. These new cytokines may have significant therapeutic potential, and they may be useful in diagnosis of inflammatory diseases. Dr. Matsushima's group includes five guest researchers, one postdoctoral fellow and one special volunteer, and collaborators include Drs. Oppenheim, E. Appella (NCI) and A. Anderson (DOD). Dr. Matsushima is the LMI expert on protein purification and characterization.

Dr. Durum, at present a Senior Staff Fellow, has studied the role of cytokines in immunity. He and colleagues have been studying how IL1 serves as a cofactor, together with antigen, in T cell activation. They have shown that IL1 stimulates the production of c-jun, whereas antigen stimulates production of c-fos. These two components come together to form the transcription factor AP-1, which in turn was shown to activate the IL2

promoter. In other cell types (non-T cells), IL1 can act alone without the need for a second signal: in hepatocytes, IL1 induced collagenase, and in pituitary cells, IL1 induced β-endorphin. In these non-T cells, IL1 induced both jun and fos. Thus, IL1 works alone in these cell types, by inducing both the jun and fos components of AP-1. They have shown that the IL1 induction of jun is transcriptional and are currently studying the promoter for jun, to identify the IL1 responsive element. These studies will contribute to our basic understanding of how cytokines act on target cells.

Dr. Durum and colleagues are also studying the process of rearrangement of the genes for the antigen receptors in T lymphocytes: what are the stimuli that induce the pre-T cell to rearrange, what are the nuclear factors that cut and splice the DNA, where does this process occur in the body, and when does it occur during development? A new PCR technique was developed to study this, and used to show that the gene rearrangement occurs earlier in development than previously thought, and more importantly, that it can occur outside the thymic environment. Two important types of gene rearrangements occur, the physiological rearrangement of antigen receptor genes and the pathological rearrangement of oncogenes, thus understanding this process is fundamental to understanding not only immunity but oncogenesis as well. A major new project in Dr. Durum's laboratory will be in creating transgenic mice that lack specific cytokine genes; such mice will be used for purposes of examining the complex in vivo roles of individual cytokines. This approach uses the technique of homologous recombination in embryonic stem cells. Dr. Durum's group includes one post-doctoral fellow and two guest researchers, and collaborators include T. Williams and J. Kant (U.Penn), M. Karin (UCSD), P. Herrlich (W. Germany), J. Axelrod and M. Fagarasan (NIMH). Dr. Durum is the LMI expert on the role of cytokines in immunity.

Dr. Louis Matis will join the Office of the Chief in the summer of 1990, replacing Dr. Howard Young. Dr. Matis is an established investigator in the immunological fields of T cell activation and development. These aspects of the immune response have not been previously represented in the LMI, which has focused more on cytokines and non-specific elements of immunity and inflammation.

IMMUNOBIOLOGY SECTION

Dr. Luigi Varesio, acting head of the Immunobiology Section, studies how macrophages are activated (by various agents, including IL2, IFNγ and endotoxin) to kill tumor cells. He and colleagues have shown that IL2 activates macrophages via one subunit of the IL2 receptor, the p75 subunit, without a requirement for the the other subunit, p55. IL2 induces not only tumor killing by this pathway, but also induces expression of c-fms, which is the receptor for the cytokine CSF-1; the consequence is that IL2-activated macrophages can recognize CSF-1, which prolongs their ability to kill tumor cells.

Dr. Varesio and colleagues have shown that IFNγ activates macrophages to kill tumor cells by a pathway quite different from IL2 or endotoxin. Macrophages ordinarily express c-fos constitutively. Whereas IL2 or endotoxin activation do not affect fos expression, IFNγ activation strongly

inhibits this constitutive fos expression. These findings suggests two equally novel possibilities, that the fos gene promoter has stimulatory elements that are blocked by IFNγ, or that it has negative regulatory elements that are activated by IFNγ. Another aspect of the mechanism of IFNγ action has been found: picolinic acid is formed and mediates several of the events triggered by IFNγ in macrophages.

Dr. Varesio's group has shown that endotoxin, like IL2, activated macrophages to express c-fms. However, endotoxin also induced other responses, including the enzyme 2'5'oligoA synthetase. Another endotoxin response found in macrophages was the formation of a nuclear factor that bound to a site on the LTR of HIV - this nuclear factor may play a role in the replication of the virus in monocytes, and could also be involved in activating endogenous macrophage genes during endotoxin stimulation of macrophages to kill tumor cells. Dr. Varesio's group includes four postdoctoral fellows and two guest workers, and his collaborators include Drs. R. Wiltrout (LEI), E. Blasi (Perugia, Italy), D. Radzioch (Montreal) and D. Longo (OAD). Dr. Varesio is the LMI expert on macrophages and interferon.

LYMPHOKINE SECTION

Dr. Francis Ruscetti, head of the Lymphokine Section, directs research in two major areas: first, the role of cytokines in hematopoiesis, and second, the biology of retroviruses (HTLV and HIV). In the area of hematopoiesis, recent studies have concerned roles of the cytokine TFGβ. His group has shown that TGFβ had several powerful effects on hematopoiesis. The net effect in vivo was that TGFβ strongly suppressed hematopoiesis by reversibly blocking early stem cells from entering cell cycle. From in vitro analyses, different stages and lineages of hematopoietic cells showed different responses to TGFβ. The earlier stages of stem cells (HPP-CFU, CFU-GEMM and BFU-E) were growth inhibited by TGFβ, whereas later stages were not inhibited, or were even enhanced in growth rate. The mechanism of TGFβ inhibition occured both by down-regulating the number of receptors for positive growth factors, as well as through other inhibitory mechanisms.

Dr. Ruscetti and colleagues have also made various observations regarding roles of TGFβ in neoplasia. Leukemic cell lines could be divided into two groups, those that were growth inhibited by TGFβ, and those that were not. They have evidence that neoplastic B cells may grow by escaping from a TGFβ mediated autocrine inhibitory loop. They have also shown that phorbol ester, which inhibits the growth of some tumor cells, can do so by inducing production of TGFβ and expression of its cell surface receptors.

Dr. Ruscetti's studies of the retroviruses HTLV-1 and HIV-1 have recently focused on the question of viral latency: how is expression of these viruses naturally suppressed in certain cells? Understanding how viral expression is naturally suppressed could suggest new treatments for the diseases mediated by these viruses. Examining HTLV-1 in the lymphoid cells from infected patients, both T and B cells contained integrated provirus, but whereas the T cell transcribed the viral genome, the B cell did not.. The provirus from the B cell was not defective, since it could be reactivated in a T cell. Since the HTLV-1 LTR was activated by its own

transactivating factors, they tested whether providing exogenous transactivators could activate the latent virus in the B cell; transactivation did not activate the latent virus, indicating that the B cell either has a deficiency of positive cofactors that are required to work on the LTR together with the transactivator, or alternatively that the B cell has negative regulators.

A similar picture emerged from Dr. Ruscetti's studies of HIV-1 latency. While the HIV-1 virus was productive in T cells, macrophages maintained the virus in a latent state. Two mechanisms appeared to operate in this latency. Negative regulation of the HIV-1 LTR occurred in the macrophage; this negative regulation occurred in part because the macrophage nucleus contained factors that interfered with the binding of the transcription factor NFkB to its site on the viral LTR. The second mechanism may involve methylation of the LTR. Dr. Ruscetti heads a group consisting of a guest researcher and four postdoctoral fellows, and his collaborators include Drs. Oppenheim (LMI), Longo (OAD), Gooey and Wiltrout (LEI), Urba, Rossio, Gonda, and Keller (PRI), Twardzik and Purchio (Oncogen,Inc), Ellingsworth (Collagen, Inc.), Derse (LVC), Poiesz (Upstate Medical Center), and Kung (LBP). Dr. Ruscetti is the LMI authority on hematopoiesis and retroviruses.

CYTOKINE MOLECULAR MECHANISMS SECTION

Dr. William Farrar, head of the Cytokine Molecular Mechanisms Section, has studied the molecular mechanisms of action of several cytokines, including IL2, IL3 and GM-CSF. IL2 and other hematopoietic cytokines were shown to regulate tyrosine phosphorylation in vivo. Although the receptors for these cytokines do not contain intrinsic kinase domains, the data suggested that they are in a tight association with one or more tyrosine kinases. Dr. Farrar and colleagues have developed specialized methods to isolate in vitro the tertiary structure of the IL2 receptor subunits and the kinase responsible for the transmembrane signal. These methods will allow the biochemical purification and subsequent molecular cloning of this receptor specific protein kinase.

Additionally, Dr. Farrar's group have identified novel structures involved in intracellular signalling. They have molecularly cloned five new tyrosine kinases from human leukemic cell mRNA which may be overexpressed in some human leukemic cell types. They will continue to characterize these genes and their potential role in leukemogenesis. They have also identified a transcriptional regulatory element found within the promoter region of the IL2Rα gene and the homologous element in the HIV-1 LTR. This protein was purified, and found to be under the control of a cytoplasmic inhibitor. The activation of this protein was inhibited by the immunosuppressant cyclosporin. Dr. Farrar's group includes four postdoctoral fellows and one special volunteer, and his collaborators include Drs. Wahl (NIDR), Clause (Georgetown U.), Beach (Cold Spring Harbor) and Howard, Kelvin and Evans (PRI).

INTERRELATIONSHIPS BETWEEN LMI INVESTIGATORS

Cytokines form the thematic base of the LMI. While many other areas of research are also performed here, the LMI has become one of the major

centers for cytokine research, and this reputation attracts many visiting scientists and postdoctoral fellows to work here. Each investigator is known for an expertise that has contributed to the reputation of the LMI in cytokine research, briefly summarized as follows. Dr. Oppenheim is a pioneer in cytokine research and attracts many of the scientists here. Dr. Matsushima has identified new inflammatory cytokines. Dr. Durum is known for the immunology of cytokines. Dr. Varesio is a contributer in cytokines and anti-tumor immunity. Dr. Ruscetti is an expert in hematopoietic cytokines. And Dr. Farrar is an authority on intracellular mechanisms of cytokine action. LMI scientists are involved in many outside activities related to cytokine research, including organizing meetings, editing books and journals, teaching courses, giving lectures, consulting, and serving on study sections.

There are many regularly scheduled meetings of the scientists in the LMI. Each of the LMI investigators hold weekly group meetings. A number of journal clubs operate. All LMI investigators and staff come together once a week to present research in progress, report on scientific meetings or hear an invited lecturer. In addition, there are weekly BRMP-wide invited lectures, staff meetings, and annual conferences 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 BRMP also performs clinical research through Clinical Research Branch (CRB), and a monthly meeting is held that brings together the clinical investigators with the basic scientists. This provides a forum that enables LMI investigators 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09289-05 LMI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Restorative Role of Cytokines in Hematopoiesis and Oncogenesis

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
	F. Ruscetti	Senior Investigator	LMI, NCI
	K. Matsushima	Visiting Scientist	LMI, NCI
	K. Hirose	Guest Researcher	LMI, NCI
	N. Colburn	Chief	CBS, LVC, NCI

COOPERATING UNITS *(if any)*
Armed Forces Radiobiology Research Institute, Bethesda, MD (R. Neta);
Program Resources, Inc., Frederick, MD (F. DeBenedetti, C. Faltynek, R. Hornung)

LAB/BRANCH
Laboratory of Molecular Immunoregulation

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	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.)*

Pre-administration of cytokines, in particular IL 1 can protect mice from lethal doses of irradiation ranging from 900 to 1,200 cGy. IL 1 when administered after irradiation can protect mice up to doses of 1,000 cGy. 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). Long term survivors were chimeric and tolerant to recipient and donor alloantigens, but exhibited immunological competence. These data suggest that IL 1 may prove clinically useful in patients undergoing bone marrow transplantation.

The role of cytokines in oncogenesis was also investigated. JB6 is a nontumorigenic murine epidermal cell line which undergoes irreversible phenotypic modification when treated with tumor promoters, such as PMA. JB6 loses anchorage dependence, as measured by colony formation in soft agar, and becomes tumorigenic in nude mice. The induction of transformation by PMA is inhibited by the addition of antipromoters, such as retinoids. TGF-β also was able to decrease, in a dose dependent manner, the number of colonies induced by PMA. This effect was not due to nonspecific inhibition of growth, since TGF-β did not inhibit the growth rate of untreated and PMA treated adherent JB6 cells. IFNα-β, IL 6 and IL 1 had no significant effect on colony formation induced by PMA. Moreover, TGF-β enhances the antipromoting activity of suboptimal concentrations of retinoic acid. Our data suggest that TGF-β may play a role in the regulation of transformation of JB6 cells.

PROJECT DESCRIPTION

PERSONNEL

Joost J. Oppenheim	Chief	OC, LMI, NCI
Dan L. Longo	Associate Director	OAD, NCI
Francis W. Ruscetti	Senior Investigator	LS, LMI, NCI
Kouji Matsushima	Visiting Scientist	OC, LMI, NCI
Kunitaka Hirose	Guest Researcher	OC, LMI, NCI
Nancy Colburn	Chief	CBS, LVC, NCI

MAJOR FINDINGS

The purpose of these studies is to delineate the protective (pretreatment) and restorative (posttreatment) effects of cytokines in countering the toxic bone marrow suppressive effects of irradiation and chemotherapy in murine animal models. The second purpose of our studies is to identify those cytokines with differentiative effects that can counteract the in vitro transforming effects of tumor promoters such as phorbol myristic acetate (PMA).

Radioprotective Effects of Cytokines

In collaboration with Dr. Ruth Neta, we previously reported that prior adminstration of IL 1 and TNF, but not GM-CSF, G-CSF, IL 2 or IFNα,β have radioprotective effects and can protect mice from lethal doses of irradiation ranging from 900 to 1200r. 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, and 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.

We have recently shown that administration of antibodies to IL 1 receptors (35F5 directed against p80) to mice inhibits the radioprotective effect of LPS by about 75%. Similarly, administration of anti TNFα antibodies also blocks much of the radioprotective effect of LPS. Administration of both of these antibodies together with LPS actually results in a radiosensitizing effect. The treated mice are killed by lower doses of irradiation than normal mice. This indicates that induction of IL 1 and TNF by LPS are responsible for the radioprotective effects of LPS and that LPS in addition must be inducing another cytokine(s) with radiosensitizing effect which remains to be identified. Furthermore, administration of anti-IL 1 receptor antibodies to normal mice also has a radiosensitizing effect suggesting either that low levels of IL 1 that are generated by continual stimulation in our conventional environment contribute to our normal resistance to radiation damage or alternatively the antibodies

interfere with the restorative effects of IL 1 that is known to be induced as a consequence of radiation damage.

Although both GM-CSF and G-CSF by themselves 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 and the fact that IL 1 induces CSF suggest that these cytokines may act in concert on in vivo 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 improved survival of lethally irradiated mice. IL 1-treated, as compared to non-treated mice by five days after irradiation had greatly enhanced numbers of nucleated bone marrow cells and increased endogenous splenic colonies suggesting that IL 1 administration stimulates bone marrow hematopoiesis.

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 ugm of IL 1 were needed to protect mice from 800-1000 irradiation as compared with the 100-500 ng 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 also 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.

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 ug 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 C57B1/6 (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 - 6 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 boné marrow cells. Our 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 106 bone marrow cells. 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 1,360 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 up to 6 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 months 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.

The mechanism by which IL 1 and TNF may be radioprotective has been investigated further by testing the hypothesis that the induction of mitochondrial manganese superoxide dysmutase (MnSOD) may contribute to radioprotection. The level of MnSOD activity in a number of cell lines did not correlate with the level of radioresistance. However, transfection of cell lines performed by Drs. K. Hirose and K. Matsushima with plasmids containing the cDNA for MnSOD increased their radioresistance, as well as their ability to resist toxic doses of chemotherapeutic agents of the type that generate reactive oxygen intermediates (ROI). Conversely, transfection of cell lines with anti-sense cDNA for MnSOD yielded cell lines that were more radiosensitive and more susceptible to the toxic effects of chemotherapeutic drugs that generate ROI. These studies do support the notion that induction of MnSOD by IL 1 and TNF may contribute to the radioprotection by these cytokines. On the other hand, we did

observe that adrenalectomized mice could be radioprotected by IL 1. This indicate that induction of glucocorticoids by IL 1 TNF does not account for radioprotective by these cytokines.

The other major problem that we have investigated is the possibility that cytokines may exert direct antitumor effects through their differentiation promoting activities. This possibility was investigated in collaboration with Dr. N. Colburn (CBS, LVC, NCI), Drs. F. DeBenedetti and C. Faltynek (LBP, NCI). It is well established that the immortal murine JB6 epithelial cell line when treated with PMA or other tumor promoter undergoes an irreversible transformation and becomes able to grow in an anchorage independent manner as measured by increased colony formation in soft agar and becomes tumorigenic in nude mice. This induction of transformation by PMA is inhibited by a variety of antipromoters including retinoids, glucocorticoids and SOD. The antipromoter activity of a number of reconbinant cytokines such as IL 1, IFN, IL 6, TNF and TGFβ was tested and only TGFβ was active in counteracting the in vitro effect of PMA on JB6 cells. The possibility that TGFβ mediates the effect of retinoic acid (RA) was investigated and RA did not induce TGFβ production, but upregulated the expression of receptors for TGFβ on JB6 cells. However, since anti TGFβ did not block the antipromoter effects of RA and synergized with RA in blocking PMA effects, the mechanism of antipromoter activity of these two agents is distinct. These findings suggest that TGFβ may have an antitransforming activities on epithelial cells.

Conversely, TNFα stimulated in a dose dependent manner, anchorage independent growth of both JB6 and NIH 3T3 cell lines. Concentrations of TNFα in the nontoxic range enhanced colony formation in soft agar about eight fold. PMA and TNFα acted in synergy. The effects of TNFα were irreversible suggesting that TNF may have tumorigenic consequences for epithelial cells.

PROPOSED COURSE

Since therapeutic doses of IL 1 have considerably greater side effects in man than G-CSF and GM-CSF, combinations of these cytokines are being tested further to establish the optimal ratios of lower doses of IL 1 and G-CSF or GM-CSF with radioprotective effects. For the same reason the radioprotective effect of "ex vivo" incubation of donor bone marrow cells for various lengths of time with IL 1 with or without other cytokines is being more thoroughly explored. The capacity of IL 1 to interact with other radioprotective agents such as anti asialo GM-1 and WR 2721 is being investigated with Dr. J. Ortaldo and we are collaborating with Dr. R. Wiltrout in studies of the hematopoietic restorative effects of IL 1 in mice being treated with chemotherapeutic agents.

Although studies of in vitro tumor promotion have implicated several cytokines that may have antipromoting in the case of TGFβ or promoting effects in the case of TNF, this needs to be confirmed with in vivo assays. Furthermore, it is important to identify, isolate and purify other cytokines with such activities.

SIGNIFICANCE

Our results suggest that IL 1 may be a clinically useful hematopoietic factor.

Our in vitro data also suggests that other cytokines may play a role in controlling the growth of transformed cells.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09216-10 LMI

PERIOD COVERED: October 1, 1989 through September 30, 1990

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
	K. Pulkki	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*: Program Resources, Inc., Frederick, MD (G.L. Gusella, T. Musso); LEI, BRMP, DCT, NCI (R. Wiltrout); Instituto di Microbiologia Medica, Perugia, Italy (E. Blasi); McGill Univ., Montreal, Canada (D. Radzioch); OAD, NCI-FCRF (D. L. Longo).

LAB/BRANCH: Laboratory of Molecular Immunoregulation

SECTION: Immunobiology Section

INSTITUTE AND LOCATION: NCI-FCRDC, Frederick, Maryland 21702-1201

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.)*

The cytokine network and molecular events regulating the activation of macrophages, monocytes and Kupffer cells to express tumoricidal activity were investigated. We focused on the analysis of the cytokines IL 1, IFN γ, IL 6, CSF 1, of the receptors for IL 2 and CSF 1 and of endotoxins. We demonstrated that both macrophages and monocytes express the p75 subunit of the IL 2 receptor. IL 2 could fully activate Kupffer cells and monocytes whereas IL 2 functioned only as a costimulatory agent, together with IFNγ, in inducing cytotoxic murine macrophages. Moreover, IL 2 regulated the expression of the c-fms oncogene (the CSF 1 receptor) in human monocytes but not in murine macrophages. We also demonstrated that increased CSF 1 receptor expression was important for prolonged expression of tumoricidal activity by monocytes. Studies on the response of macrophages to IFNγ, revealed that the expression of c-fos, an early activation gene, is inhibited by treatment of monocytes with IFNγ but not with other activating agents such as IL 2 or endotoxins indicating that gene expression in response to IFNγ may involve modulation of nuclear transactivating factors. Studies on the biochemical events associated with the response of macrophages to IFNγ revealed a connection between tryptophan metabolism and macrophage activation. We have shown that picolinic acid, a tryptophan catabolite, which is produced by macrophages mediates some of the molecular events that follow the interaction between macrophages and IFNγ. These findings have important clinical applications. Studies on the response of macrophages to endotoxins revealed a complex mechanism of gene expression that affects the levels of the enzyme 2'5' oligoA synthetase and of c-fms mRNA. Moreover, endotoxins elicited the expression of nuclear transactivating factors that bind the LTR of the HIV virus and are of major potential relevance for the replication of the virus in monocytes as well as for the coordinate control of gene expression during macrophage activation.

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
Kari Pulkki	Visiting Fellow	IS, LMI, NCI

OBJECTIVES

The objectives of the research have been to study: 1) the cytokine network and the molecular events leading to the activation of human monocytes and murine macrophages to express tumoricidal activity; 2) regulation of expression and function of lymphokine receptors (IL 2 and CSF-1) on human monocytes and murine macrophages; 3) control of the expression of nuclear transactivating factors binding the LTR of the HIV-1 retrovirus and; 4) the role of tryptophan metabolites on the activation of macrophages by IFNγ.

MAJOR FINDINGS

1. Characterization of Interleukin 2 Receptor Expression and Function on Murine Macrophages

It is now well established that human monocytes express functional IL 2 receptors (IL 2R). However, there is little or no information about the expression of IL 2R, or the influence of IL 2, on human macrophages, murine monocytes or murine macrophages. Although they are closely related, monocytes and macrophages are distinct populations of cells that might very well differ in their expression of IL 2R or their responses to IL 2. Therefore, this project was undertaken to examine several aspects of IL 2R expression and function on either murine peritoneal macrophages or immortalized murine macrophage cell lines that were established and characterized in this laboratory.

We utilized a model system of murine macrophage cell lines (ANA-1 and GG2EE) that was established by infecting normal murine bone marrow-derived cells with the J2 (v-raf/v-myc) recombinant murine retrovirus. ANA-1 macrophages did not constitutively express detectable levels of mRNA for the p55, α-chain, subunit of the IL 2R (IL 2Rα). However, a brief exposure (2-5 hrs) to gamma-interferon (IFNγ) was sufficient to induce IL 2Rα mRNA in ANA-1 macrophages. ANA-1 macrophages that were treated with IFNγ for 18-24 hrs did not express IL 2Rα mRNA. Flow cytometric analysis indicated that ANA-1 macrophages expressed low constitutive levels of IM 1$\Sigma\beta$. Affinity binding and cross-linking of [$1^2{}_5{}^I$]IL 2 to ANA-1 macrophages demonstrated that IL 2Rα and the p70-74, β-chain, subunit of the IL 2R (IL 1Rβ) were both constitutively present on ANA-1 macrophages. IFNγ increased the expression of IL 2Rα on ANA-1 macrophages but did not increase the expression of IL 2Rβ on these macrophages.

In contrast to results obtained with human monocytes, IL 2 alone did not induce the tumoricidal activity of ANA-1 macrophages or thioglycollate-elicited mouse peritoneal macrophages. However, IL 2 acted synergistically with IFNγ to induce the tumoricidal activity of murine macrophages. That IL 2 was able to synergive with IFNγ for the induction of tumoricidal activity in the C3H/HeJ mouse derived GG2EE macrophage cell line or C3H/HeJ-derived peritoneal macrophages indicates that IL 2 can circumvent the genetic defect of the LPS-hyporesponsive C3H/HeJ mouse. This finding suggests that IL 2 may play a role as a physiological counterpart of endotoxin/LPS in vivo.

The results of these studies demonstrate the expression of IL 2R on murine macrophage cell lines and normal murine peritoneal macrophages and establish the role of IL 2 as a co-stimulator of macrophage-mediated tumoricidal activity. Moreover, our results indicate major differences in the expression and function of the IL 2R proteins found on murine macrophages versus human monocytes. In general, our data support the hypothesis that the differentiation of the monocyte into the macrophage is associated with an alteration in the expression and function of the IL 2R on these cells.

We have utilized the polymerase chain reaction to examine the effects of IL 2 on gene expression in murine macrophages. Preliminary data indicates that IL 2 alone or in combination with IFNγ can induce or augment the expression of specific mRNA for colony stimulating factor-1, IL 6 and the JE "early competence" gene.

Although 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 immortalized murine macrophages.

2. cDNA Cloning of a Potentially Novel Gene in Macrophages that is Partially Homologous to the Murine IL 2 Gene

Utilizing Northern blot analysis and the polymerase chain reaction it was observed that macrophages express an IFNγ inducible mRNA that is partially homologous to the murine IL 2 gene. A cDNA library has been developed from the mRNA of ANA-1 macrophages stimulated with IFNγ and is currently being screened for the IL 2-like gene. The cloned cDNA will soon be isolated and sequenced to determine if it represents a new and novel gene in mouse macrophages.

3. Human Monocyte Activation and c-fms Expression

The c-fms proto-oncogene, a marker for cells of the myelomonocytic lineage, codes for the macrophage colony stimulating factor receptor (CSF-1R). CSF-1 is found in relatively high levels in human serum suggesting that the regulation of CSF-1 activity in human monocytes might occur at the receptor level. We investigated the effects of IL 2 and IFNγ, two potent monocyte activator, on the expression of c-fms. We found that IL 2 but not IFNγ selectively enhances c-fms expression (mRNA and glycoprotein levels). Since c-fms codes for CSF-1R, we postulated that human monocytes treated with IL 2 could display a different sensitivity to CSF-1. We tested this hypothesis and found that CSF-1 sustains

IL 2 but not IFNγ induced cytotoxicity in human monocytes. Our results may have implications in the design of clinical protocols employing adoptively transferred monocytes into cancer patients.

4. Human Monocyte IL 2 Receptor

The ability of human monocytes to respond to IL 2 indicates the presence of a functional IL 2R on these cells. However, fresh human monocytes do not express Tac antigen and there are no reports concerning the expression of p75 on fresh or IL 2 treated monocytes. We investigated the expression of IL 2 receptor (IL 2R) subunits in human monocytes utilizing the TU27 monoclonal antibodies (mAb), which recognizes the p75 chain and anti-Tac mAb, which recognizes the p55 moiety of the IL 2R. We found that p75 but not p55 is constitutively expressed in more than 90% of fresh human monocytes. Antibody to p75 but not to p55 inhibited the activation of monocytes to a cytotoxic stage induced by IL 2. Our data demonstrate that the p75 chain is expressed on human monocytes and is involved in the activation of monocytes by IL 2. Preliminary results obtained with Northern blot analysis suggest that IL 2 differentially affects mRNA expression of the IL 2R subunits.

5. Effect of IFNγ on the Early-Response Gene, fos

Expression of the early response gene, c-fos, has been linked to differentiation and activation in macrophage/monocytes. Our previous studies have indicated, however, that expression of c-fos is more closely associated with signal transduction, as through PK-C, than with the functional outcome of cytotoxicity in these cells. In monocytes, we find no increase in c-fos after activation of IL 2 or IFNγ. On the contrary, IFNγ inhibits expression of c-fos induced by FCS, CSF-1, or endotoxin. The inhibitory effect of IFNγ can be seen within 30 min of exposure to these stimuli. This strong and rapid inhibitory effect of IFNγ on fos gene expression suggests a mechanism by which this lymphokine can effectively modulate a cascade of later gene expression.

6. Effect of IL 2 on Rat Liver Macrophages

We have previously shown that IL 2 is an effective independent activator of human monocytes, but requires a costimulus in the activation of murine peritoneal macrophages, or of the murine v-myc/v-raf-immortalized mature macrophage cell line, ANA-1. We have now developed a model system to study mature rat liver macrophages. Kupffer cells are isolated from the liver by collagenase perfusion, centrifugal elutriation, and adherence. These cells are not spontaneously cytotoxic, but can become cytotoxic in vitro in response to rhu-IL 2 to an extent as great or greater than that seen with endotoxin and IFNγ, or in rat monocytes. Preliminary studies show that they do express receptors for IL 2. These findings indicate that differences in species, or sources of tissue macrophage, may result in different responses to IL 2; and further indicate that IL 2 may have an important, and previously under-estimated, role in the immune response of the liver Kupffer cells.

7. Molecular Mechanism Involved in the Control of the Expression of 2'5' OligoA Synthetase (OASE) in Murine Macrophages

Both LPS and IFNγ can induce OASE mRNA in the murine macrophage cell line ANA-1 although LPS appears to be a much stronger stimulator. When used in combination, LPS plus IFNγ prevented the accumulation of OASE mRNA to levels induced by these stimuli used individually. The costimulation also produced a decrease in the enzymatic activity of OASE compared to untreated control cells. The use of anti-IFNα/β antibodies almost completely abrogated LPS induction of OASE mRNA indicating that LPS stimulation was mediated by secreted IFNα/β which acted in an autocrine manner. However, combined treatment of ANA-1 with IFNα/β and IFNγ did not result in a decrease OASE mRNA level suggesting that LPS is also activating a mechanism involved in the negative control of OASE induction. Both transcriptional and posttranscriptional mechanisms are involved in LPS induced accumulation of OASE mRNA. Particularly, the half life of OASE mRNA, measured after the block of transcription with actinomicyn D, appears to be about 8 hr in LPS treated cells compared to 4 hr in the control cells.

OASE can be activated by double stranded structure formed by rRNAs. rRNA accumulation has been demonstrated to be associated with the induction of the cytotoxic state in murine macrophages. In order to investigate the possible effects of the constitutive expression of OASE in macrophages, we have engineered a retroviral vector suitable for the infection of a mature murine macrophage cell line tested for the susceptibility to transforming retrovirus. Experiments of macrophage infection are in progress.

8. Effects of IL 6 and IL 4 on the Expression of Tumoricidal Activity by Human Monocytes

Various cytokines induce IL 6 secretion from many cell types. IL 6 has not been reported earlier to induce or modify tumoricidal activity by monocytes. In our test system, using a pure (elutriated) population of monocytes, we found that IL 6 has a synergistic effect with IL 2 in inducing cytotoxicity. There was no effect on IFNγ induced cytotoxicity by IL 6. Further studies will elucidate the mechanism of this modulatory effect.

There are many reports of the inhibitory action of IL 4 on several functions of inflammatory cells. Using human monocytes, we found that IL 4 inhibits IL 2 induced cytotoxicity. IL 4 did not have any effect on the IFNγ induced tumoricidal activity. Further studies are needed to elucidate the role of IL 4 as an inhibitor of monocyte-mediated cytotoxicity in vivo.

9. Activation of Macrophages by IFNγ: The Tryptophan Connection

Picolinic acid, a metobolite of tryptophan, is a costimulator of macrophage-mediated tumoricidal activity. In vitro, it synergizes with IFNγ and activates mouse macrophages to express maximal tumoricidal activity. Also hyporesponsive mouse macrophages (C3/HeJ) can be activated to express tumoricidal activity with the combination of IFNγ and picolinic acid. However, we did not find any effect to the tumoricidal activity of human monocytes. The latter fact may indicate a correlation between the function (tumoricidal activity) and the stage of differentiation of monocyte-macrophages. To study this hypothesis, an ion-

pairing HPLC method using tetrabutyl ammonium as a counter-ion was developed to measure picolinic acid secretion by macrophages in culture.

10. Effect of Cytokines on HIV-LTR in a Human Promonocyte Cell Line, U937

Experiments begun last year were completed to study the effects of cytokines on expression of HIV-LTR in monocytoid cells. The HIV-LTR was linked to the chloramphenicol acteyl transferase marker gene and integrated after transfection into a human promonocyte cell line, U937. Endotoxin and TNFα were found to independently induce expression of HIV-LTR. GM-CSF or TNFα were additive with endotoxin in enhancing that expression. This is the first report that cytokines can affect HIV-LTR in the absence of transactivating factor in monocytic cells. It is also the first indication that endotoxin can act alone, and that BRMs can be additive, in their effects on HIV-LTR.

11. Expression of HIV-LTR Binding Proteins in Macrophages

HIV-1 LTR contains sequences in which multiple regulatory elements recognized by cellular nuclear factors are clustered. Two regulatory regions can be distinguished in the HIV-1 LTR depending on their enhancer or inhibitory effects on viral expression. Many of the nuclear factors potentially binding to these regulatory regions have been identified. The enhancer region contains three binding sites for the cellular transcriptional factor Spl and two eleven base pairs segments homologous to a sequence present in the enhancer region of the immunoglobulin k gene binding the nuclear factor NFkB. The negative regulatory region (NRE) contains three AP1-like binding sites target of the fos-jun complex and a negative regulatory factor binding sites target of a protein identified in the HeLa cells and of a 50 kDa protein present in T cells where it is involved in controlling the expression of the IL 2α receptor. Since monocytes and macrophages can serve as a reservoir for the HIV-1 and a vehicle for the dissemination of the virus we analyzed different regions of the HIV-1 LTR for their ability to bind nuclear proteins from GG2EE murine macrophages cell lines that have been immortalized from mouse bone marrow. In the initial studies a 219 bp fragment of the NRE of the HIV-1 LTR containing the two known proteins binding sites (AP1 and NRF) was used as a probe for detecting nuclear DNA binding proteins by band shift assay. The 219 bp fragment generated two major shifted bands when reacted with the nuclear extract of untreated GG2EE cells demonstrating the constitutive expression of different DNA binding proteins recognizing the NRE in these cells. Activation of GG2EE cells with LPS (10 ug/ml) induced the expression of a third shifted band of intermediate mobility relative to the migration of those expressed in control cells. The LPS responsive factor was detectable after six hrs of LPS stimulation. Cycloheximide selectively inhibited the induction of the LPS responsive factor without affecting the expression of the constitutive bands. To define the region recognized by the LPS responsive factor the 219 bp fragment was divided in three fragments of 123, 58 and 38 bp by digestion with the restriction enzymes Hpa II and Ava I and each fragment was tested in band shift experiments. When reacted with the nuclear extracts from untreated GG2EE cells, the 123 bp fragment, containing the AP2 like sites, generated only one shifted band which was similar in intensity and mobility to that obtained with the nuclear extract from LPS-treated GG2EE cells. In contrast when the same nuclear extracts from untreated or LPS treated GG2EE cells were reacted with the 58 bp fragment, a

different pattern of shifted bands was observed. The nuclear proteins of LPS-treated macrophages generated two major shifted bands as opposed to the extract of control cells where one major shifted band was observed. The 38 bp fragment did not generated detectable shifted bands. These results provide the first evidence that GG2EE macrophages expressed constitutive nuclear proteins binding the NRE of HIV-1 LTR and that they responded to LPS stimulation with the induction of an LPS-responsive factor, different from fos-jun complex, binding the 58 bp fragment.

HIV-1 can infect human monocytes and it has been shown that LPS protect monocytes from productive infection possibly through an inducible host cell mechanism. These results are consistent with the possibility that binding of the LPS responsive factor to NRE may be involved in inhibiting the ability of HIV to infect endotoxin activated monocytes. It is also been reported that HIV production by infected monocytes can be further augmented by endotoxin stimulation suggesting that binding to the HIV-1 LTR of the LPS responsive factor in conjunction with the HIV coded regulatory proteins may overcome the inhibitory effect of NRE on viral expression.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09260-08 LMI

PERIOD COVERED: October 1, 1989 through September 30, 1990

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
	L. Thompson	Visiting Fellow	LMI, NCI
	M. Shiroo	Special Volunteer	LMI, NCI
	A. Samanta	Guest Researcher	LMI, NCI
	C. Zachariae	Guest Researcher	LMI, NCI
	Y. Mahe	Guest Researcher	LMI, NCI
	N. Mukaida	Guest Researcher	LBP, NCI
	A. Hishinuma	Guest Researcher	LMI, NCI

COOPERATING UNITS *(if any)*

NCI, Bethesda, MD (E. Appella); DOD, Ft. Detrick, Frederick, MD (A. O. Anderson).

LAB/BRANCH: Laboratory of Molecular Immunoregulation

SECTION: Immunobiology Section

INSTITUTE AND LOCATION: NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	.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.)*

Studies of the signal transduction mediated by 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 kinase 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 l-plastin. Tumor necrosis factor and PMA which have many over-lapping biological activities with IL 1 also activate the same serine kinase and phosphorylate the identical cytosolic proteins. The IL 1/TNF/PMA responsive promotor region of IL 8 gene was localized to the region -94 to -71 bp, which consists of C/EBP and NFkB-like factor binding elements. The nuclear factors bound to these elements may also be similarly phosphorylated and activated by IL 1/TNF/PMA activated serine kinase. 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 physiochemical properties of these molecules have been determined 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
Lorraine Thompson	Visiting Fellow	IS, LMI, NCI
Masahiro Shiroo	Special Volunteer	IS, LMI, NCI
Ajoy Samanta	Guest Researcher	IS, LMI, NCI
Claus Zachariae	Guest Researcher	IS, LMI, NCI
Yann Mahe	Guest Researcher	IS, LMI, NCI
Naofumi Mukaida	Guest Researcher	LBP, NCI
Atsushi Hishinuma	Guest Researcher	IS, LMI, 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 properties of IL 1/TNF inducible newly defined cytokines, IL 8 and MCAF.

ACCOMPLISHMENTS

1. Signal transduction mediated by post IL 1-receptor molecular events: Identification of cell-associated proteins phosphorylated in response to stimulation with IL 1 and responsible protein kinase.

We previously reported the identity of the receptor for IL 1α and IL 1β, 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 reported that recombinant human IL 1α at as low a concentration as 1 ng/ml induces predominantly the phosphorylation of 65 and 74 kDa cytosolic proteins in human PBMC. The phosphorylation occurs within 1 min and reached a maximum by 10 min after 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.

The identical cytosolic 65 and 74 kDa proteins are phosphorylated in human PBMC after stimulation with TNF or phorbol esters, but not with diacylglycerol, prostaglandins E, or forskolin. 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. The molecular mass of this protein kinase has been estimated to be ~25 kDa on HPLC

gel filtration. This partially purified protein kinase (p65 kinase) is located in the cytosol and distinct from protein kinase A, C or casein kinase in substrate specificity and appears to participate in signal transduction by IL 1, TNF and PMA.

2. Analysis of the regulation of human IL 8 gene by IL 1, TNF and PMA.

The production of IL 8 is not constitutive. IL 8 is induced in a variety of cell types by stimulation with mitogens and cytokines. Among cytokines, only IL 1 and TNF are known to induce the IL 8 gene expression at the transcriptional level. Transfection of a human fibrosarcoma cell line with CAT expression plasmids linked to a 5'-flanking deletion mutants of the IL 8 gene demonstrated that the nucleotides between -94 and -71 bp from the start of the first exon are essential and sufficient for IL 8 gene induction by either IL 1, TNF or PMA. This sequence is composed of two cis-elements; one is the potential binding site for an NF-kB-like factor and the other for a C/EBP-like factor. Mutations in either elements abolished IL 1, TNF, and PMA responsiveness. This finding provides the first evidence that cooperation between two distinct cis-elements may be required for induction of gene expression by either IL 1 or TNF.

3. Biological and molecular characterization of human IL 8 and MCAF.

Infiltration 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 have been reported to be directly chemotactic for human neutrophils, monocytes, and T lymphocytes, we have recently purified neutrophil, monocyte, and lymphocyte chemotactic factors to homogeneity from the conditioned media of human PBMC or a monocytic cell line stimulated with mitogens and have shown that these chemotactic factors are structurally 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. The three dimensional structural analysis of recombinant IL 8 by both nuclear magnetic response and X-ray crystalography has shown that IL 8 exists as dimer and IL 8 dimer has a structural motif in which top of a six-stranded anti-arallel β-sheet platform derived from two three-stranded Greek keys, one from each monomer unit. Biologically active IL 8 has been chemically synthesized, and expressed in E. coli, and CHO cells. The entire 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 reaches maximal expression 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 accumulation, whereas higher doses (10 ul of 100 ng/ml/site) predominantly induced neutrophil infiltration. 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 x 10^{-10}M and a molecular mass of ~60 kDa. 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.

The regulation of IL 8 receptor expression by the IL 8 ligand was examined using freshly isolated human peripheral blood neutrophils. IL 8 down-regulated >90% of its own receptor expression within 10 min at 37°C. This down-regulation was associated with internalization of the ligand. The radiolabeled IL 8 molecules after internalization were proteolytically degraded, and trichloroacetic acid-soluble molecules were released into the culture medium starting at 60 min. Lysosomotropic agents could inhibit this degradation of ligand suggesting the involvement of lysosomal enzymes in causing proteolytic digestion. IL 8 receptors reappeared on the cell surface within 10 min after removal of free ligands from the culture medium. Cycloheximide did not alter the reappearance of the receptor suggesting that de novo protein synthesis of IL 8 receptors is not involved in this event and that receptors are probably recycled. The addition of lysosomotropic agents partially inhibited the reappearance/recycling of the receptors, although none of these agents inhibited the binding of ligand to the surface receptors or ligand internalization. Ammonium chloride reduced the IL 8-induced neutrophil chemotactic response in a dose-dependent fashion. These data suggest that IL 8 receptor expression is dynamically regulated by IL 8 and that rapid recycling of IL 8 receptors may be essential for the chemotactic response of neutrophils.

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%). Purified unlabelled MCAF competed with ^{125}I-labelled MCAF in binding to human PBMC, whereas a similar basic heparin binding leukocyte chemoattractant, IL 8 did not, suggesting the existence of specific receptors for MCAF on human PBMC which are distinct from receptors for IL 8. 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.

SIGNIFICANCE

1. 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 all phosphorylate common 65 and 74 kDa cytosolic proteins. The 65 kDa protein has been identified to be 1-plastin which has been reported to be widely expressed in leukocytes and transformed fibroblasts. Although the receptors for IL 1, TNF and PMA are distinct, our study has shown that IL 1, TNF and PMA activate a common cytosolic serine kinase to phosphorylate the 65 kDa protein. This may explain why IL 1, TNF and PMA have many overlapping biological activities. The identified IL 1/TNF/PMA activated serine protein kinase is at least distinct from protein kinase C, protein kinase A, or casein kinase and may be crucial to signal transduction by IL 1.

2. The chromosomal human IL 8 gene has been cloned and the entire nucleotide sequence has been determined. Study of the IL 1/TNF/PMA responsive promotor in the 5'-flanking region of IL 8 gene has established that the region -94 to -71 bp, which consists of C/EBP-like and NFkB-like factor binding sites, is responsible for IL 1, TNF and PMA responsiveness. These results suggest that the protein kinase which is commonly activated by IL 1, TNF and PMA stimulation may be involved in modifying and activating nuclear factor(s) which binds and regulates IL 8 gene expression.

3. Biological, biochemical and physiochemical properties of two novel proinflammatory cytokines, IL 8 and MCAF have been elucidated. The success in making three-dimensional structural model of IL 8 will facilitate the development of novel antagonists and agonists for IL 8 and also localize the active site.

PROPOSED COURSE

1. Elucidation of signals transduced through type I IL 1R and type II IL 1R.

2. Purification and cDNA cloning of the identified IL 1, TNF and PMA activated serine kinase.

3. Identification, purification and cDNA cloning of C/EBP and NFkB like factors which regulate IL 8 gene expression.

4. Study of the modification of the C/EBP and NFkB-like factor by IL 1/TNF/PMA activated serine kinase and the effect on the in vitro transcriptional activity.

5. 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, gout and uveitis.

c. Purification of IL 8 and MCAF receptors, cDNA cloning and study of the signal transduction mechanism mediated by these receptors.

d. Modification/truncation and development of antagonistic or agonistic polypeptides based on the tertiary structural analysis of these chemotactic cytokines.

e. Establishment of the biological activities of related molecules and identification of other IL 1/TNF inducible cytokines.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09287-06 LMI

PERIOD COVERED
October 1, 1989 through September 30, 1990

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. Bristol	IRTA Fellow	LMI, NCI
	U. Csaikl	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (F. Aiello, W. Gotlieb, K. Muegge, M. Smith, F. Csaikl, M. Vila); University of Pennsylvania (T. Williams, J. Kant); UCSD (M. Karin); Kernforschungszentrum, Karlsruhe, W. Germany (P. Herrlich); LEI, NCI (H. Young); NIMH (J. Axelrod, M. Fargarasan).

LAB/BRANCH
Laboratory of Molecular Immunoregulation

SECTION
Immunobiology Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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 have been studying how cytokines induce gene expression. We previously showed that IL 1 induced IL 2 via the transcription factor AP-1. We now show that the components of AP-1 (jun and fos) are differently regulated in different cell types responding to IL 1. T cells produce only jun in response to IL 1, and need a signal from the antigen receptor to induce fos - thus IL 1 is a cofactor in T cells. Hepatocytes and pituitary cells produce both jun and fos in response to IL 1 - thus IL 1 alone is sufficient to induce many genes via AP-1 in these cells. We are examining the jun promoter for IL 1 responsive elements.

Another project deals with T cell receptor (TcR) rearrangement. We have developed a new PCR technique for studying this gene rearrangement process, and are applying it to study the cellular stimuli and intracellular mechanisms of TcR gene rearrangement. A major new direction will be targeting cytokine genes, with the goal of creating mice that lack certain specific cytokines; these will be used to study the in vivo roles of the cytokines.

PROJECT DESCRIPTION

PERSONNEL

Scott K. Durum	Senior Staff Fellow	IS, LMI, NCI
Lynn Bristol	IRTA Fellow	IS, LMI, NCI
Ulrike Csaikl	Visiting Fellow	IS, LMI, NCI

OBJECTIVES

1) How does IL 1 control the transcription of genes?

2) Regulation of TcR rearrangement.

3) Targeting cytokine genes.

METHODS EMPLOYED

1. To explore how IL 1 controlled the transcription factor AP-1, we analyzed the components of this factor, c-jun and c-fos in several cell types responding to IL 1. Messenger RNA for c-jun and c-fos was measured using Northern analysis. To directly measure transcription of the endogenous c-jun and c-fos genes in a cell, nuclei were isolated following IL 1 stimulation and "nuclear runoff" was performed. To analyze the portion of the c-jun promoter that responded to IL 1, cells were transiently transfected by electroporation with several parts of the jun promoter attached to the CAT reported gene; later the activity of the CAT enzyme was analyzed. The preceding methods were used to determine how the jun and fos genes and mRNAs were affected by IL 1. We also examined earlier events following IL 1 stimulation, asking what second messengers were involved in communicating between the IL 1 receptor and the jun gene; thus, we tested various inhibitors, stimuli and products of several second messenger systems.

2. We have developed a new method for studying the rearrangement of TcR genes using PCR. Two primers (15b) are used, one for the 5′ end of a given v-beta gene, the other for the 3′ end of a given j-β gene. In germ line DNA, these genes are too far apart for a PCR reaction to occur. However, if cells have rearranged this v-β to this J-β, the PCR reaction occurs, and we identify the product by hybridization with a probe for the 3′ end of the v-β sequence. We have tested a number of different v and j probes and are satisfied with the specificity and sensitivity of the technique. Using this method, we have examined the rearrangement of TcR genes in terms of where this occurs in the body, and when it occurs during ontogeny; our primary objective is to determine what the signals (probably cytokines) are for a pre-T cell to rearrange these genes, and then to examine the nuclear proteins actually involved in the gene rearrangement.

MAJOR FINDINGS

1. How does IL 1 control the transcription of genes? We previously noted (with T. Williams and J. Kant) that IL 1 activated the IL 2 gene via the AP-1 transcription factor. We have now analyzed the two components of the AP-1 factor, fos and jun, to determine how IL 1 regulates them. We find that in a T cell line (LBRM), IL 1 induces jun, but not fos mRNA, whereas the T cell antigen receptor induces fos but not jun; this explains why in the T cell, both IL 1 and antigen are needed to produce an active AP-1 factor. The situation is different in two non-T cell lines, HEP-G2, a hepatoma, and ATT20 (with J. Axelrod and M. Fargarasan), a pituitary line. In both of these lines, IL 1 alone (without need for a costimulus as in the T cell) is capable of inducing secretion of a specific protein (collagenase and bendorphin, respectively). In both of the non-T lines, IL 1 induces both jun and fos mRNA, not just jun as in the T cell. This explains how IL 1 acts as a direct stimulus in some cells, versus a costimulus in other cells. We previously showed that the AP-1 factor is critical to the IL 1 response, by removing the AP-1 motif from the IL 2 promoter, and showing that it no longer responded to IL 1. We have now reinforced the importance of AP-1, using anti-sense constructs (to jun and fos) to block the IL 1 induction of β endorphin in the pituitary line.

How does IL 1 cause the mRNA increase of jun (and in some cells fos): Potentially, there are two main regulatory means: increased transcription of the jun and fos genes, versus increased stability of their respective mRNAs. We conclude that the increase in transcription is the most important IL 1 control of both jun and fos, since using the nuclear runoff, IL 1 is seen to induce transcription of both jun and fos genes in HepG2 cells (we also looked for effects on mRNA stability and saw none). We then examined the promoter for jun, to try to localize the IL 1 responsive element. The jun promoter has not been extensvely studied; in the one published paper on the subject, all promoter function has been localized to a 130bp fragment 5′ of the transcriptional start site. However, we find that this fragment (from M. Karin) is constitutively very active in HepG2 cells, and it is not further activated by IL 1. We are therefore testing the provocative possibility (with P. Herrlich) that a silencing element occurs upstream of this fragment, and that IL 1 acts by turning off this silencer.

What are the biochemical signals leading from the IL 1 receptor to the jun promoter? We have tested various chemical agents that are used to implicate different second messenger pathways. We find that the PKC pathway, though it can activate the jun promoter, does not seem to mediate IL 1's action. The PKA pathway also does not seem to be involved in IL 1's action. We have very preliminary evidence that a tyrosine kinase is involved, based on use of a tyrosine kinase inhibitor.

2. Our interest in TcR rearrangement came from our findings that different cytokines induced pre-T cells to express different TcR genes. We thought this could mean that different cytokines were inducing rearrangement of different TcR genes. However, using a sensitive new PCR technique, we find that in our previous experiments, the pre-T cells (thymic "double negative" cells) had already rearranged their TcR genes, and that the particular cytokines we used had the effect of inducing transcription of these already rearranged genes. We

have now set out to determine at what stage the pre-T cell actually rearranges these genes (is it in the thymus, or is it prior to the thymus). Eventually we will explore the stimuli that induce the rearrangement, and the nuclear factors that recombine the DNA. Our major finding thus far regards the anatomical location where pre-T cells rearrange. It has been generally assumed the rearrangement of TcR genes occurs in the thymus, but the evidence has been indirect. Thus far, we have evidence that TcR rearragement can definitely occur outside the thymus (it may also occur in the thymus, we do not know this yet). Thus nude mice, lacking a thymus, have rearranged cells, and fetal liver contains rearranged cells before a thymus develops.

SIGNIFICANCE

1. IL 1 has many effects on many cell types. Our laboratory was the first to implicate the AP-1 transcription factor in the actions of IL 1 (or any cytokine). We continue to pursue this mechanism, which continually reveals novel aspects of the IL 1 pathway, from new second messengers to new regulatory sites on the jun gene. Hence, these studies, while directed at explaining the mechanism of action of an important cytokine (IL 1), have also yielding new information on controls of a key transcription factor, AP-1.

2. In studying the TcR rearrangement process, our main progress thus far has been in developing a new PCR technique. We used this technique to make the unexpected finding that TcR genes could rearrange outside the thymic environment, which was the site previously thought to be where T cells rearrange. Although the thymus may nevertheless prove to be the major site of rearrangement, it is clear from our findings that the thymic microenvironment is not unique in providing pre-T cells with the right signals to rearrange. This should help us identify what these signals are. Gene rearrangement is fundamental to immunity, but very little is known about the process, primarily because it has not been amenable to study in vitro; it is our hope that our new approach, because of its sensitivity, will allow us to study this process. What we learn about gene rearrangement in immune cells may shed light on carcinogenesis, which often involves gene rearrangements.

3. Targeting cytokine genes exploits an exciting new technology that will allow us to assess the in vivo roles of these molecules. Only a few genes have been successfully targeted, and we look forward to discovering many unanticipated roles of the cytokines.

PROPOSED COURSE

1. The jun promoter will be the principal focus of our next studies on the mechanism of gene action of IL 1. Our working hypothesis is that there is an important regulatory site upstream of the previously defined jun promoter; this site would act as a silencer (in some cells), and the effect of IL 1 would be to release the silencer. To test this, we have obtained cAT constructs (from P. Herrlich) containing both the previously identified (130bp) jun promoter, as well as large stretches upstream of this. We will test whether this construct is silent in IL 1-responsive cells, and is activated by IL 1. If so, we will test various truncations and internal deletions to try to localize an IL 1-responsive silencer. Concurrently, we will look for nuclear proteins that bind

to the upstream region. This will be done by synthesizing a variety of oligonucleotides that cover this region, then identifying proteins that bind to them; we would look for proteins influnced by IL 1, either positively or negatively. If such sites and their binding proteins are identified, the motif will be compared with other known elements. If it looks novel, the means are available at the BRMP to clone the genes for the proteins that bind the motif.

2. Our primary goals are to identify the stimuli that induce a pre-T cell to rearrange its TcR, then to pursue the nuclear proteins that actually recombine the DNA. Our immediate goals are to induce the rearrangement process in vitro. We are thus using various sources of prospective pre-T cells (bone marrow from nude mice and fetal liver) and are stimulating these cells with various combinations of cytokines (IL 1-8), lectins and other stimuli, and various preparations of accessory cells. We hope this will lead us to the stimuli that induce rearrangement. To examine the nuclear events, we will then seek homogeneous pre-T cells (possibly hybridomas) that can be similarly induced to rearrage. We will then pursue the proteins that bind DNA in the regions involved in recombination; techniques such as band shifting, DNA footprinting and transfection of expression libraries into the pre-T cell will be used to try to isolate, characterize and clone the relevant proteins.

3. A major future project will be aimed at producing mice which lack particular cytokine genes; these will be used to assess many aspects of the biology and pathology of cytokines. This involves a new approach, gene targeting, involving the technique of homologous recombination. Defective cytokine genes will be constructed, then used to replace the functional cytokine genes in embryonic stem cells. These embryonic stem cells with defective cytokine genes will then be used to produce mice which will thus have their normal cytokine genes replaced by the defective cytokine genes. Many studies will then be performed to determine the effect of deleting a cytokine on the physiology, and immune and inflammatory responses. We will first target the IL 1α, β and receptor genes by homologous recombination techniques. The resulting mice, if they survive, will be extensively studied for abnormalities. We cannot anticipate the many possible consequences of IL 1 deletions (there are a multitude of reported IL 1 effects), but they should at least include effects on the immune and inflammatory processes. If mice fail to survive, or die in utero, we will explore the pathologies.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09251-08 LMI

PERIOD COVERED: October 1, 1989 through September 30, 1990

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-FCRDC, Frederick, Maryland 21702-1201

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 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 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 <u>trans</u> 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. For instance we have found that negative regulation of chronic expression in monocytes is mediated through inhibition of binding of the transcription factor, NF-kB to the HIV enhancer.

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. Restricted and Latent Expression of HIV-I in Human Monocytes Occur by Different Mechanisms

In monocytes infected with HIV-I and HIV-II, we can 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 restricted 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. The 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.

II. 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-1 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, transactivation, viral RNA and proteins were absent. 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.

III. Negative Regulation of Chronic HIV-1 Expression in Monocytes: Involvement of the 65+50 kD NF kB Heterotetramer

The clinical latent period of AIDS is influenced by factors that stimulate human immunodeficiency virus (HIV) replication in several different cell types. Although monocytic cells can provide a reservoir for viral production in vivo: 1) their regulation of HIV transcription can be either latent; 2) restricted; 3) or productive. These differences in HIV gene expression have not been molecularly defined. In THP-1 cells with restricted HIV expression, there is an absence of DNA binding complex formation with the HIV-1 promoter-enhancer leading to markedly less production of viral RNA. This absence of binding was localized to the NF-kB region of the HIV enhancer with the 65+50 kD NF-kB heterotetramer being completely lost. Addition of purified NF-kB protein to nuclear extracts from cells with restricted expression overcomes this lack of binding. In addition, treatment of these nuclear extracts with sodium deoxycholate (DOC) restored their ability to form the heterotetramer suggesting the presence of a specific inhibitor of the activity of NF-kB. Furthermore, nuclear extracts from these cells with restricted expression treated with lipopolysaccharide (LPS) leads to increased viral production and increased NF-kB activity. Thus, both NF-kB binding complexes are needed for optimal viral transcription. The binding of 65+50 kD heterotetramer to the HIV-1 enhancer can be negatively regulated in monocytes providing one mechanism leading to restricted HIV gene expression.

IV. Cytotoxic Effector Mechanisms are Active Against Cells Productively Infected by HIV-I and HTLV-I but not against Restricted Infected Cells

AIDS is due to 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, the 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.

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 the 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 any significant differences in the biological functions or surface markers were altered following 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 hybridization 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.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09254-08 LMI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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
	D. M. Linnekin	NRC Fellow	LMI, NCI
	G. G. Garcia	Special Volunteer	LMI, NCI
	J. V. Snider	IRTA Fellow	LMI, NCI

COOPERATING UNITS *(if any)*
National Institute of Dental Research, NIH (L. Wahl); Georgetown University, (K. Clouse) Cold Spring Harbor Laboratories, (D. Beach) Program Resources, Inc., Frederick, MD; (Z. Howard, D. Kelvin, G. Evans).

LAB/BRANCH
Laboratory of Molecular Immunoregulation

SECTION
Cytokine Molecular Mechanisms Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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.)*

The laboratory has actively investigated the molecular mechanisms of action of several human cytokines including, IL 2, IL 3, and GM-CSF. IL 2 and other hematopoietic cytokines were shown to regulate tyrosine phosphorylation in situ. Although the receptors for these cytokines do not contain intrinsic kinase domains, the data suggested that they are in a tight association with one or more tyrosine kinases. We have developed specialized methods to isolate in vitro the tertiary structure of the IL 2 receptor subunits and the kinase responsible for the transmembrane signal. These methods will allow the biochemical purification and subsequent molecular cloning of this receptor specific protein kinase. Additionally, we have molecularly cloned five new human tyrosine kinases from human leukemic cell mRNA which may be overexpressed in some human leukemic cell types. We will continue to characterize these genes and their potential role in leukemogenesis. The laboratory has identified a transcriptional regulatory element found within the promoter regions of the IL 2Rα gene and the homologous element in the Human Immunodeficiency Virus-1 long terminal repeat. This protein was purified, and found to be under the control of a cytoplasmic inhibitor. The activation of this protein in situ was inhibited by cyclosporin A.

PROJECT DESCRIPTION

PERSONNEL

William L. Farrar, Jr.	Senior Investigator	CMMS, LMI, NCI
Dennis F. Michael	Visiting Fellow	CMMS, LMI, NCI
Anna T. Brini	Visiting Fellow	CMMS, LMI, NCI
Diana M. Linnekin	NRC Fellowship	CMMS, LMI, NCI
Gonzalo G. Garcia	Special Volunteer	CMMS, LMI, NCI
James V. Snider	IRTA Fellow	CMMS, LMI, NCI

OBJECTIVES

The laboratory is conducting research in two areas: 1) the discovery of protein kinases associated with hematopoietic growth factor receptors with specific emphasis on their role in cytokine receptor triggering and whether mutations in these proteins occur in neoplastic hematopoietic cells, and 2) the biochemical mechanisms of "Enhancer" binding protein activation in an effort to learn more about gene regulation in normal leukocytes and enhancer proteins which regulate the transcription of HIV-1.

MAJOR FINDINGS

I. Isolation and Characterization of Tyrosine Kinases Associated with Cytokine Receptors

The last few years investigators have focused on identifying the receptors for the cytokines IL 2, IL 3, and GM-CSF. All of these cytokines are in clinical trials and their receptors have been molecularly cloned. The analysis of these receptor cDNA sequences have revealed that they comprise a new family of receptors which has not been structurally seen previously in cell biology termed the "hematopoietin superfamily". Unlike other characterized growth factor receptors, none of these receptors contain any known catalytic domains, suggesting that they use a novel mechanism to transduce signals initiated by ligand binding. We have developed novel assays and methods to isolate cytokine receptor complexes in vitro which permits us to study multimeric receptor components and identify associated protein kinases. Based on these new methodologies we are proceeding with the purification and molecular cloning of a tyrosine kinase associated with the human IL 2 receptor complex.

Using the polymerase chain reaction we have molecularly cloned five new tyrosine kinases not previously identified. We are in the process of completly sequencing the cDNAs of these kinases and determining whether they are expressed at high levels in various myeloid and lymphoid leukemias. The cloned kinases have several structural properties similar to those of known oncogenes and may therefore act as potential oncogenic proteins in human cell transformation.

IL 2 was shown to regulate the phosphorylation and activity of two important cellular proteins, p34CDC2 kinase and the retinoblastoma gene product RB. The

activation of the nuclear CDC2 kinase was initiated by cell cycle dependent dephosphorylation and the CDC2 kinase did not phosphorylate the RB gene product in situ. The RB gene product stands out in importance since it has anti-oncogenic properties and has been found to complex with DNA tumor virus proteins such as E1A and large T antigen. We examined whether the RB protein formed a complex with the pX40 transforming protein of a human retrovirus HTLV-I and found that there was no complex formation. This indicated that RB may not participate in cellular resistance to this type of transforming virus.

We have confirmed using murine and human cell models that the cytokines IL 2, IL 3, and GM-CSF are using some of the same protein kinase pathways and that the same tyrosine kinase is associated with each of their unique receptors. We are isolating the kinases themselves and several of their substrates for eventual purification and subsequent molecular cloning.

II. Studies on the Regulation of the IL 2 Receptor α Gene and HIV-1 Transcription.

One of the consequences of receptor triggering is the stimulation of de novo gene transcription. This process is due in part to the activation of "enhancer" binding proteins via the activation of protein kinases. Therefore, one principal target of protein kinase cascades are DNA binding proteins. We have identified a genetic transcriptional regulatory region common to both the IL 2Rα gene and the LTR of HIV-1. The protein, termed NF-kappaB (NF-kB), was purified from PMA-induced Jurkat cells and found to be a 47 kDa protein that bound with high affinity to the homologous sequences present in both genes. The activation of this protein could be inhibited with the immunosuppressive agent cyclosporin A. Several cytokines activate the enhancer binding protein and upregulate gene expression of HIV-1 or the IL 2Rα gene. The NF-kB protein was found to be under the control of an inhibitor and was activated in vitro with mild detergents or denaturants. We developed a modified assay which allowed the detection of this protein and other DNA binding proteins in very small numbers of living cells.

Other regulatory "enhancer" like sequences were also observed in the HIV-1 LTR. These other regulatory regions were silent in some cells and active in T cells with less mature characteristics. These data suggested that several different regulatory regions of the HIV-LTR may be used depending on the state of differentiation of the host cell.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09264-08 LMI

PERIOD COVERED: October 1, 1989 through September 30, 1990

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
	S. E. Jacobsen	Visiting Fellow	LMI, NCI
	J. J. Oppenheim	Chief	LMI, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*: LEI, BRMP, DCI, NCI (H. Gooey, R. Wiltrout); Program Resources, Inc., Frederick, MD (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-FCRDC, Frederick, Maryland 21702-1201

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. Primitive hematopoietic cells such as high proliferative potential colony forming cell (HPP-CFU) and CFU-GEMM and BFU-E were inhibited by TGF β in vitro. Thus, the ability of TGF β to block hematopoietic cell growth depends on the state of differentiation of the cell. In contrast, addition of TGF β to GM-CSF stimulated bone marrow cells greatly augmented growth, leading predominantly to an increase in granulocytes. In vivo experiments with TGF β show that the hematopoietic stem cells are reversibly prevented from entering the cell cycle. This growth inhibitory action functions on at least two levels: 1) trans-down modulation of the cell surface receptors for positive regulatory signals and/or interfering with post-receptor signalling of these molecules. Leukemic cell lines could be either sensitive or insensitive to TGF β mediated growth inhibition. Growth of neoplastic B lymphocytes can occur by escaping from a TGF β mediated autocrine inhibitory loop. Activation signals (e.g. phorbol esters) can inhibit tumor cell growth by stimulation of active TGF β production and induction of cell surface expression of functional TGF β receptors. These results suggest that TGFβ may have utility as a bone marrow protective and as an anti-tumor agent.

PROJECT DESCRIPTION

PERSONNEL

Francis W. Ruscetti	Senior Investigator	LS, LMI, NCI
Maria C. Birchenall-Sparks	Guest Researcher	LS, LMI, NCI
Lydia A. Falk	IRTA Fellow	LS, LMI, NCI
Claire M. Dubois	Visiting Fellow	LS, LMI, NCI
Sten E. Jacobsen	Visiting Fellow	LS, LMI, NCI
Joost J. Oppenheim	Chief	OC, LMI, NCI
Dan L. Longo	Associate Director	OAD, NCI

MAJOR FINDINGS

I. Direct Inhibitory Effect of TGF β on Primitive Hematopoietic Cell Growth Hydrolysis Correlates with Activation of T-Cell Specific Genes

We have previously shown that TGF β selectively inhibits both human and murine hematopoietic progenitor cell growth. In all these assays, we have observed that TGF β1 and TGF β2 are equipotent inhibitors of hematopoiesis. As a result of the pleiotropic nature of TGF β, it is possible that these effects were indirect. To study this, 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 when 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 enriched in primitive hematopoietic stem cells. 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 both of these stem cells with HPP-CFU potential is also inhibited by TGF β.

II. TGF β: A Trans-Down Modulator of Cell Surface Expression of Cytokine Receptors on Hematologic Cells

The mechanisms by which TGF β acts as a potent inhibitor of the growth and functions of lymphoid and hemopoietic progenitor cells are not known. Cell proliferation depends not only on the presence of growth factors, but also on the development of specific receptor-signal transducing complexes. We therefore

investigated whether the inhibitory actions of TGF β could be mediated by inhibition of growth factor receptors. TGF β inhibited the constitutive level of interleukin-1 receptor (IL 1R) expressed on several murine lymphoid and myeloid progenitor cell lines as well as IL 1R expression induced by interleukin 3 (IL 3) on normal murine and human bone marrow cells. Furthermore, treatment of bone marrow progenitor cells with TGF β concommitantly inhibited the ability of IL 1 to promote HPP colony formation and also blocked IL 1-induced IL 2 production by EL 4 6.1 cells. These findings provide the first evidence that the inhibitory action of TGF β on the growth and functional activities of hematopoietic and T cells is associated with a reduction in the cell surface receptor expression for IL 1.

Since TGF β is a potent inhibitor of the stimulatory effect of many hematopoietic growth factors, in addition to IL 1, we examined the effect of TGF β on cell surface receptor expression for CSF. TGF β inhibits the expression of receptors for granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL 3), and granulocyte-CSF (G-CSF) receptors on both murine factor-dependent and independent hematopoietic progenitor cell lines without a significant change in receptor affinity. A maximum reduction in GM-CSF receptor numbers of 65 to 77% was observed by 96 hrs incubation with TGF β. The TGF β induced trans-down-modulation of GM-CSF receptors was prolonged, non-cytotoxic but reversible, and not due to endogenous production of GM-CSF. The TGF β induced reduction in CSF receptor numbers preceded TGF β's growth inhibitory action. In addition, the $ED5_0$ (1 to 10 pM) for TGF-β's CSF receptor modulatory and antiproliferative effect was similar. The effect of TGF-β on cell surface CSF receptor expression was specific, since the expression of other cell surface proteins (Ly 5 and Ly 17) was not affected by TGF β treatment. These data suggest that TGF β down-regulates the growth of hematopoietic progenitor cells by reducing the expression of growth factor receptors.

III. Stimulation of Granulopoiesis by TGF β: Identification of a Novel Hematopoietic Progenitor Cell

TGF β1 selectively inhibits the growth of immature hematopoietic progenitor cells while more mature lineage restricted progenitors are not inhibited. In contrast, in the presence of saturating concentrations of granulocyte-macrophage colony stimulating factor (GM-CSF), TGF β promotes a 3- to 5-fold increase in the number and size (>.5 mm) of bone marrow colonies in soft agar in a dose-dependent manner with an ED50 of 10 to 20 pM. TGF β1 alone exerts no growth stimulatory or inhibitory effect. Morphological examination of colonies demonstrate an increase in granulocyte colonies. In suspension culture, TGF-β1 and GM-CSF stimulates an increase in total viable cells with markedly enhanced neutrophilic differentiation and a concomitant decrease in the number of monocytes-macrophages by day 6 in culture. Limiting dilution analysis demonstrate a 2- to 5-fold increase in the frequency of progenitor cells that respond to GM-CSF plus TGF-β1 vs GM-CSF alone. Bone marrow progenitors recovered from mice after a 2 day treatment with 5-fluorouracil (5 FU) responded to a combination of GM-CSF and TGF β1 while either factor alone had no effect. TGF β upregulated the number of GM-CSF receptors on bone marrow cells cultured in GM-CSF. Thus, TGF β1 can act as a bifunctional mediator of hematopoietic cell growth, and TGF β1 and GM-CSF act together to stimulate granulopoiesis as

measured by large granulocyte colony formation, tentatively designated burst-forming unit granulocyte.

IV. TGF β1 Receptor Modulation During Hematopoietic Cell Growth and Differentiation

TGF β1 belongs to a family of polypeptides with multifunctional effects on hematopoietic and nonhematopoietic cells and has been shown to bind three distinct receptor types (Type I: 53, Type II: 73-90, and Type III: 250-350 kDa). In this study, chemical cross-linking of radiolabeled TGF β1 was used to study the regulation of these receptors during hematopoietic cell differentiation. Freshly isolated human monocytes and neutrophils expressed predominantly the 65 kDa receptor species. In addition, murine resident and thioglycollate-elicited, peritoneal exudate macrophages expressed all three receptor types. Expression of TGF β1 receptors during hematopoiesis showed that freshly isolated, granulocyte-depleted, murine and human bone marrow cells exhibited little or no specific TGF β1 receptor expression. However, culturing of human bone marrow progenitors in GM-CSF or IL 3 for 7 days resulted in development of mature granulocytes and monocytes which expressed predominantly the 65 kDa TGF β1 receptor. Examination of the development and modulation of TGF β1 receptors on mature macrophages showed that murine bone marrow progenitors cultured in CSF-1 or GM-CSF for 3 days generated adherent macrophages which preferentially expressed the 65 kDa binding protein. By day 7, the expression of all three TGF β1 receptor species was increased on murine CSF-1-derived macrophages but not on GM-CSF-derived macrophages. In addition, IFN-gamma treatment resulted in a dose and time-dependent down-modulation of receptor expression on peritoneal exudate macrophages. The results indicate that freshly isolated hematopoietic cells exhibit differential TGF β1 receptor expression. Furthermore, as bone marrow mononuclear cells mature in vitro, they acquire TGF β receptor expression which can be subsequently down-modulated by activation agents. These data suggest that receptor modulation by growth factors or activation/differentiation agents results in a potential feedback loop by which cellular responses to TGF β1 are controlled.

V. Growth and Differentiation of Human Myeloid Leukemic Cells: Role of TGF β

HL-60, a promyelocytic leukemic cell line, whose growth and differentiation is not affected by TGF β, possesses few detectable functional TGF β receptors. Thus, we examined the effect of TGF β alone and in combinations with other factors on the growth and differentiation of the human promyelocytic cell line HL60 and the human monoblastic cell line U937. Treatment with TGF β alone did not significantly affect growth or differentiation of HL60 cells, while it significantly inhibited proliferation and induced monocytic differentiation of a small percentage of U937 cells. Combinations of TGF β and tumor necrosis factor α (TNF α) acted in synergy to inhibit cell proliferation and to induce monocytic differentiation of both HL60 and U937 cells. In contrast, no synergy was observed when HL60 cells were treated with TGF β in various combinations with interferon α (IFN α), interferon-gamma (IFN-gamma), and retinoic acid. Examination of TNF α receptor expression on HL60 and U937 cells showed that these cell lines expressed comparable levels of high-affinity TNF α binding sites. Treatment of HL60 and U937 cells with TGF β did not induce significant changes in TNF α receptor expression in either cell line. In contrast, HL60

cells expressed much lower levels of TGF β receptors than did U937 cells. Treatment of both HL60 and U937 cells with TNF α induced a dose dependent increase in expression of TGF β receptors, suggesting that the synergy between TNF α and TGF β may result, at least in part, from upregulation of TGF β receptor expression by TNF α.

Since retinoic acid (RA) has been shown to cause remission in patients with acute promyelocytic leukemia, the effect of RA on HL-60 was studied. Treatment of HL-60 with RA for 7 days has previously been shown to inhibit proliferation and stimulate granulocytic differentiation. Untreated HL-60 cells which had low levels of TGF β receptors on the cell surface showed a dose dependent increase with RA. Moreover, RA treatment resulted in a dose-dependent increase in both TGF β1 steady-state mRNA expression and treated for seven days with suboptimal concentrations of HL-60 (0.1 nM) resulted in a marked decrease in cell proliferation with no effect on differentiation suggesting that RA stimulates a TGF β1 mediated antiproliferative loop during HL-60 differentiation.

VI. Growth Inhibition of Human Neoplastic B Cells: Induction of a TGF β Mediated Autocrine Negative Loop by Phorbol Esters

Since escape from negative regulators such as TGF β could play a role in the growth of neoplastic cells, we examined the effects of TGF β on lymphoid leukemic cells. TGF β exerts profound inhibitory effects on a number of cell types, including normal B and T lymphocytes. In contrast, we have found a number of lymphoid tumor cell lines to be insensitive to the anti-proliferative effects of TGF β1 or TGF β2. Binding and cross-linking with radioiodinated TGF β1 demonstrated either low or absent expression of all three TGF β receptor species on 3 B cell tumor lines but T-cell and non-T, non-B tumors expressed large numbers of receptors. Treatment of the B-cell lines with phorbol 12-myristate-13acetate (PMA) induced the expression of TGF β receptors and inhibited proliferation in all three lines in a dose- and time-dependent manner. The cell lines constitutively produced TGF β mRNA and released small amounts of latent TGF β, however, PMA induced increased expression of TGF β mRNA and, more importantly, release of active TGF β. A neutralizing antibody to TGF β was able to reverse the PMA-induced growth inhibition and addition of exogenous TGF β reversed the effects of the neutralizing antibody. Thus, TGF β can inhibit human lymphoma cell growth in vitro through an autocrine mechanism. Some lymphoma cells appear to have escaped from TGF β negative regulation by failing to express functional TGF β receptors and/or failing to secrete active TGF β. One mechanism by which PMA acts to inhibit lymphoma cell growth is by inducing the expression of TGF β receptors and the secretion of active TGF β thereby reestablishing an autocrine growth inhibitory loop.

VIII. 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 meyloid cell line 32D. Consistent with this observation is the expression of the TGF β1 65 kD type I 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 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.

In addition, growth factor-independent 32D-src and 32D-abl cell lines, established by infecting the interleukin-3-dependent myeloid precursor cell line (32D-123) with retroviruses containing the src or abl oncogenes, were used to study transcriptional regulation of TGF β1 mRNA. Analysis of different TGF β1 promoter constructs regulated by $pp60v^{-src}$ indicated that sequences responsive to high levels of src induction contain binding sites for AP-1. Both src and serum induced expression of the c-fos and c-jun genes in myeloid cells, resulting in transcriptional activation of the TGF β1 gene. Our results demonstrate that serum treatment increases TGF β1 mRNA levels in 32D-123 cells and suggest that the v-src protein satisfies the serum requirement by binding the AP-1 complex to the TGF β1 promoter, thereby mediating the induction of TGF β1 transcription.

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SUMMARY REPORT

LABORATORY OF EXPERIMENTAL IMMUNOLOGY

October 1, 1989 through September 30, 1990

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: Leukocyte Differentiation Section (LDS), Dr. John R. Ortaldo, Acting Head; the Cellular and Molecular Immunology Section (CMIS), Dr. Howard A. Young, Head, who replaced Dr. Craig W. Reynolds after his departure in 1988; 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. In-depth studies are performed on cell-mediated immune effector mechanisms, molecular biology of lymphokine gene structure and expression, lymphokine biological activities, monoclonal antibodies (MoAb), 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 MoAb probes and to develop new MoAb 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 MoAb 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 MoAb 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 (TNF-α), interferon (IFN-α/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 IFN-α, IFN-γ, LT or TNF failed to neutralize the cytotoxic activity observed with NKCF preparations. To assist in the characterization and purification of NKCF, mouse MoAbs 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 MoAbs do not inhibit TNF-α, IFN-α/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 MoAbs. Biochemical analysis of labeled NKCF-containing supernatants indicate that the major protein recognized by these anti-NKCF MoAbs is approximately 12,000 kD. These anti-NKCF MoAbs 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 IFN-γ from purified populations of CD3- LGLs. To initiate IFN-γ 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 IFN-γ 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 IFN-γ 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 IFN-γ in the presence of an anti-TAC (p55) MoAb. 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 MoAb (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 molecular 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 cell-mediated immunity, with emphasis on T cell and large granular lymphocyte specific regulation of gene expression, utilizing IFN-γ as a model system; 2) to study the control of lymphocyte activation at the molecular level; and 3) to study the mechanisms of tumor cell resistance, including resistance to immunological and chemotherapeutic 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 IFN-γ gene expression. This gene has been chosen because expression of IFN-γ is restricted to two cell types, T cells and CD3- LGLs. In a transfected murine T-cell line, human IFN-γ genomic DNA gene expression can be enhanced by phorbol esters or IL-2 while in a murine B-cell line, which never expresses murine IFN-γ, 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. This tissue specific region, 5′ to the coding sequence, may contain both enhancer and repressor protein binding regions. The second enhancer region, which does not appear to be tissue specific and is located in the first intron, may also be involved in enhanced gene expression in response to calcium flux. We have also now identified a human LGL-like cell line and a human B-cell line which both express endogenous IFN-γ in response to specific stimuli. The LGL cell line spontaneously produces IFN-γ and expression can be increased by PMA or IL-2. Interestingly, cyclosporin A, a potent inhibition of IFN gene expression in peripheral blood lymphocytes, enhances IFN-γ gene expression when utilized in combination with either IL-2 or PMA in this cell line. Aberrant IFN expression in also observed in the B-cell lines. Although B cells have never been reported to express IFN-γ, two B-cell lines express this protein in response to teleocidin, a protein kinase C activator. Current studies are focused on identifying the biochemical signalling events which contribute to the aberrant IFN-γ gene expression in these cell lines.

A second experimental approach towards understanding the regulation of cytokine/lymphokine gene expression has involved an analysis of the effects in vivo and in vitro 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 and in vitro in order to elucidate the mechanisms by which it enhances IL-2 therapy of a murine kidney tumor model system. We have determined that FAA rapidly induces expression of mRNA for a number of cytokines in vivo and in vitro 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 is likely to be the result of its ability to directly induce cytokine gene induction. Furthermore, specific cell populations which express the cytokine mRNA in response to FAA have now been identified. Efforts will be concentrated on identifying those regulatory regions of the cytokine genes which are required for enhanced gene expression in response to FAA and analyzing the effects of FAA on human peripheral blood lymphocytes in vitro.

The CMIS, in collaboration with Dr. John Ortaldo, has also begun studies on a unique gene which functions as a receptor on NK cells (LGL) for attachment to their tumor target. This gene, recently molecularly cloned in our laboratories, is located on chromosome 3, codes for a mRNA of 7-8kb and is expressed only in T cells and LGL. The gene contains a unique structure in the 5′ extracellular region which suggests that it may have a cis-trans isomerase activity. Currently studies are focused on defining conditions which alter mRNA expression and on the effects of introducing cDNA expression vectors containing portions of the cDNA into appropriate lymphocyte cell lines in order to evaluate structure-function relationships.

We have also identified a gene, perforin, which shows increased mRNA expression in resting human peripheral blood T cells upon treatment with high dose IL-2. Further analysis of the T-cell population has shown that the perforin gene is preferentially induced in CD-8+ T cells but is constituitively expressed and not inducible in LGL. We have also found that the levels of IL-2 needed to induce

perforin gene expression can be lowered by up to two logs when IL-6 is added to the culture medium. Additional studies have indicated that the expression of the perforin mRNA correlates closely with the cytotoxic activity of the cells.

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. Immunohistochemical analysis of human tissues and tumors with MoAb to GST-π has provided evidence that it may be useful as a preneoplastic marker in human cervical and colon cancers. In addition we have now determined that the observed increased levels of GST-π expression in cervical tissue samples can be closely correlated with the specific type of human papilloma virus DNA present in the samples.

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 in vivo, 2) to study the biological mechanisms by which these agents function, and 3) to study the hematological and chemoprotective effects of selected cytokines.

A major emphasis of the section has been to develop and utilize experimental models of chemoimmunotherapy (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 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. 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 TNF-α and IFN-γ. 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 both murine renal and colon cancers. The contribution of FAA to this combination appears to be at least partially cytokine mediated since in collaboration with Drs. Howard Young (CMIS) and LouAnn Eader (Biological Carcinogenesis and Development Program, Program Resources, Inc.), we have shown that FAA potently upregulates the expression of genes for IFN-γ, TNF-α, and IFN-α, and these cytokines can enhance the therapeutic effects of IL-2.

Further support for this hypothesis derives from studies where systemic alkalinization of mice, performed in a manner similar to that used for human clinical trials of FAA, inhibits the ability of FAA to induce cytokine genes and proteins and inhibits the therapeutic effects of FAA + rIL-2 against mouse renal cancer. The cellular mechanism by which FAA + rIL-2 mediates regression of murine renal cancer is dependent on the generation of tumor-specific $CD8^+$ lymphocytes, since depletion of that subset in vivo abrogated the ability of FAA + rIL-2 to mediate tumor regression. In collaboration with Dr. Kristin Komschlies-McConville (Biological Carcinogenesis and Development Program, Program Resources, Inc.), we are determining whether specific rearrangement in T-cell receptor genes accompanies the generation of such antitumor responses. This type of approach provides an experimental preclinical method for exploiting the antitumor effect of rIL-2 without expensive and complicated adoptive immunotherapy administration. Studies are also in progress to determine the mechanism by which FAA stimulates cytokine gene expression. FAA at doses $\geq$ 100 μg/ml directly stimulates mRNA for IFNs α and β, as well as TNF-α in murine spleen cells. This effect varies with the cell type studied such that FAA preferentially induces mRNA for IFN-γ and IFN-α in $CD8^+$ T cells and B cells, respectively. TNF-α mRNA is induced in all cell types studied.

Another major research 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 MoAbs, 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 over that observed with chemotherapy or immunotoxins alone. Cells harvested from mice that showed progressive tumor growth after chemoimmunotoxin therapy exhibited no evidence of either toxin resistance or loss of antigens from the cell surface. In vitro data has shown that IFN-γ and IFN-α 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 MoAb conjugates can result in an additive or synergistic preclinical therapeutic effect.

The ETS is also investigating 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. 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. Studies are in progress to characterize these factors. In addition, mouse endothelial cells have been isolated and grown in culture for use in studying the localization of NK and LAK cells in vivo.

The ETS is also isolating and characterizing novel leukocyte-derived antitumor factors derived from rat NK (RNK) tumor cell lines in collaboration with Dr. Thomas Sayers (Biological Carcinogenesis and Development Program, Program Resources, Inc.). These studies have demonstrated that some extracts of the

granules obtained from the RNK tumor cells contain a constituent(s) that is growth inhibitory to a variety of tumor cells in vitro. The effect seems to be predominantly cytostatic rather than cytotoxic. Furthermore, distinct morphological changes occur in sensitive cells in addition to the growth inhibition. The observed biological activity does not appear to be due to TNF-α, TGF-β, or any other-known cytokine tested to date. Using biochemical fractionation procedures we have partially purified the cytostatic molecule which appears to be a protein of about 32 KDa in both reduced and non-reduced form. It is a heparin-binding protein that is denatured by heat treatment, but is relatively resistant to trypsin. Currently we are trying to purify the molecule to homogeneity utilizing a variety of procedures including sizing columns, ion-exchange, heparin affinity, and hydrophobic interaction chromatography. We anticipate being able to soon perform a N-terminal amino acid sequence analysis on the molecule to determine whether it is a novel structure or not. If novel we will attempt molecular cloning of the molecule.

Because cytokines are often pleiotropic in their actions we have also performed another series of studies, in collaboration with Drs. Jonathan Keller (Biological Carcinogenesis and Development Program, Program Resources, Inc.), Frank Ruscetti (LMI), and Dan Longo (OAD, BRMP) that show that the in vivo administration of rTGF-β1 can transiently arrest the proliferation of early hematopoietic stem cells in both normal mice, as well as mice whose bone marrow has been made hyperproliferative by a prior injection of 150 μg/kg 5FU. In both cases CFU-c and CFU-GEMM are inhibited by about 30%-50%, respectively. Subsequently, we have shown that both rIL-1α and or TGF-β1 have chemoprotective effects in vivo against the toxic effects of cyclophosphamide (Cy). The chemoprotective effects of rIL-1α are such that pretreatment with $\geq$ 10,000 U/day for 5-7 days protects mice from the acute lethal toxicity of high doses of both 5FU and Cy. This ability to dose escalate Cy may translate into enhanced therapeutic efficacy against murine tumors. Interestingly, some of the mice that are protected against acute toxicity of Cy by rIL-1α, exhibit late mortality due to pulmonary toxicity. Further studies are in progress to determine the mechanisms for these hematological, chemoprotective and pathological effects, and to determine to what degree this approach can be used to increase the therapeutic efficacy of chemotherapeutic drugs.

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 09247-10 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

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
	E. 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); Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (J. Rossio)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Leukocyte Differentiation Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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). Studies are proceeding to define the receptors and structures involved in NK recognition. A monoclonal antibody (MoAb) was developed against NK target antigens on K562 cells and that antibody blocked LGL binding and lysis. We also developed an anti-idiotypic antibody (anti-ID) against this MoAb 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. Utilizing this anti-ID, an expression library from CD3- LGL was screened and specific cDNA clones were isolated and sequenced. Based on the predicted protein sequence, the cDNA was found to code for a unique 1016 AA protein that consisted of several distinct structural domains. The NH2-terminal domain was 50% homologous to cyclophilin, a cyclosporin binding protein. This was followed by the idiotype region, a transmembrane domain, and a 367 AA cytoplasmic domain. Studies are presently underway to further characterize and unequivocally demonstrate the receptor nature of this unique gene.

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Joyce Frey-Vasconcells	IRTA Fellow	LDS, LEI, NCI
Robin Winkler-Pickett	Microbiologist	LDS, LEI, NCI
E. 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 MoAb to K562 tumor cell membrane glycoprotein, designated MoAb #36. MoAb #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 MoAb 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 MoAb #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 IFN-γ production. Prior studies have shown that IL-2 treatment or pre-incubation of LGLs with K562 cells resulted in the secretion of IFN-γ. Our results are consistent with the activation of an NK-R by anti-ID and have 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 110 and 140 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 MoAbs [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 immunoprecipitation of both the 110 kD and 140 kD proteins. The initial cDNA clone contained a 250 bp insert with an open reading frame coding for 80 amino acids. Neither the nucleotide nor amino acid sequence possessed any significant homology to sequences contained in the Genbank sequence database. Southern blot analyses 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 7 Kb mRNA in LGLs and a 1.6 Kb mRNA in T cells. The expression and functional importance of the 6-7 Kb and 1.6 Kb mRNA in NK cells, T 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. 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.

B. Cytotoxic Factors from RNK Cells.

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 and other cytotoxic factors. This provided sufficient NKCF to permit analysis of mouse MoAbs (AO287, AO462, and AO316) that significantly inhibit the NKCF cytolytic activity in these LGL-derived supernatants. Biochemical analysis of radiolabeled (35-S) RNK-NKCF-containing supernatants indicated that the major protein recognized by these anti-NKCF MoAbs 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 hr. These studies indicate that RNK-NKCF is a unique cytolytic molecule with some antigenic similarities to cytolysin. The inability of purification of this factor due to its inherent stability, however, has been a major problem.

Recently, a membrane associated lytic factor has been isolated from rat RNK cells. This factor has been shown to be stable, active after culture in Ca++ containing medium [unlike pore forming protein isolated from RNK granules] and have many of the characteristics of NK lysis. Present studies are underway to characterize this factor and determine if it is distinct from known cytotoxic factors.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09256-08 LE

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. E. Bere	Bio. Lab. Tech.	LEI, NCI
	J. Wine	Bio. Lab. Tech.	LEI, NCI

COOPERATING UNITS *(if any)*

Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (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-FCRDC, Frederick, Maryland 21702-1201

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 a to generate killer cells against NK-insensitive targets and secrete IFN-γ by LG In addition to LGL activity being regulated by a variety of cytokines, LGLs hav been shown to produce a variety of lymphokines (IL-1, IFN, CSF, BCGF). A proje is being conducted to investigate IFN gene expression and regulation in highly purified human LGLs and T cells. Within 1 hr of IL-2 treatment of freshly isolated human LGLs, IFN-γ mRNA secreted in the culture medium within 4-6 hr of treatment. These results indicate that with certain stimuli LGLs may be the predominant source of IFN-γ from peripheral blood lymphocytes. In addition to IL-2, MoAbs to specific surface proteins have been used to study their role of these proteins as potential regulatory elements in the activation of LGLs. The CD3- LGL represents an excellent cell type to study signal transduction leading to both modulations of cytotoxicity and gene transcription, since it represents cell type "poised" for activation and capable of responding to a single stimulu Present studies involve examining agents which modulate signal transduction of IL-2 and IFN mediated events.

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Joyce Frey-Vasconcells	IRTA	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 (LGLs) 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. 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.

Present studies involve studying the BRMs that co-regulate LAK function with IL-2. In addition, studies are planned that will address the recognition mechanism of LAK effector populations and compare these to NK cells.

B. Signaling 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 IFN-γ 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 β-chain of the IL-2 receptor, which has been demonstrated to be constitutively present on fresh, inactivated CD3- LGLs.

MoAbs 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 MoAb and the identification of additional CD2 epitopes led us to further examine their ability to regulate NK and LAK cytotoxicity and IFN-γ production. Overall, these anti-CD2s produced an approximately two-fold increase in basal LAK activity. When IFN-γ production was examined, a similar trend was seen. All of the anti-CD2s except 9.6 resulted in a small increase in IFN-γ 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 IFN-γ units, and this effect was only marginally enhanced by the addition of 9.6. In addition, anti-X11-1 again inhibited IFN-γ production when combined with 9.6.

The conclusion drawn from these data is that MoAbs 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 IFN-γ gene. We have studied the effects of 1-(5-isoquinolinesulfonyl)2-methyl-piperazine-dihydrochloride (H7) a PKC inhibitor, 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. We have continued these studies examining the tyrosine phosphorylation events in BRM activated LGL and how agents which inhibit cellular functions modulate various signaling events. We have emphasized PKC and tyrosine phosphorylation events.

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. Even if this was 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 IFN-γ 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.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09349-01 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Comparative Study of Receptor-Mediated Signaling in T Cells and NK Cells

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

PI:	J. O'Shea	Expert	LEI, NCI
Others:	I. Kennedy	Medical Staff Fellow	LEI, NCI
	J. Ortaldo	Chief	LEI, NCI

COOPERATING UNITS *(if any)*
BCDP, Program Resources, Inc. (T. Bailey); EIB, NCI (A. Weissman); BRMP, NCI-FCRDC, Frederick, MD (J. Ashwell); CBMB-NICHD (L. Samelson, R. Klausner); Howard Hughes Medical Institute, Stanford University Medical School (G. Crabtree)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Leukocyte Differentiation Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, MD 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	1.8	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.)*

Recently it has become known that the T-cell antigen receptor is coupled not only to phosphoinositide hydrolysis but to a non-receptor protein tyrosine kinase (PTK) as well. We therefore investigated the role of PTK in receptor mediated signaling in T lymphocytes and NK cells. We observed that in T cells, expression of the constitutively active tyrosine kinase, v-src, resulted in constitutive IL-2 production. This means of IL-2 production is inhibitable by cyclosporin A and elevation of intracellular cAMP levels but not by depletion of protein kinase C. The mechanism of v-src regulation of the IL-2 gene is currently under investigation but may be mediated by NFkappa B. A second consequence of expression of v-src is constitutive phosphorylation of the zeta chain of the TCR. This provides a model to begin to investigate the functional importance of zeta as a tyrosine phosphorylated substrate. Isolation of v-src-expressing mutant T cells demonstrated that the induction of IL-2 was independent of zeta expression, consistent with the interpretation that following activation of a tyrosine kinase, zeta may no longer be required. Studies are underway to determine the effect of constitutive zeta chain phosphorylation on TCR coupling to phospholipase C and phosphoinositide hydrolysis.

Like the T-cell receptor, the NK FcR (CD16) is also associated with the zeta chain. To determine if the NK FcR is also coupled to a tyrosine kinase, we investigated ligand-induced zeta chain phosphorylation in NK cells. We observed that perturbation of the FcR but not the IL-2 R resulted in tyrosine phosphorylation of zeta. Preliminary evidence suggests that a kinase may be associated with zeta. Studies are underway to characterize this kinase and delineate the means by which it is regulated.

PROJECT DESCRIPTION

PERSONNEL

John J. O'Shea	Expert	LEI, NCI
Ian Kennedy	Medical Staff Fellow	LEI, NCI
John R. Ortaldo	Chief	LEI, NCI

OBJECTIVES

The overall objective of this project is to delineate the biochemical steps involved in receptor-mediated signaling in T cells and NK cells. The two prototypic receptors used for these studies are the T-cell receptor (TCR) and the Fc receptor (FcR). Surprisingly, these receptors seem to share a number of characteristics. Both of these receptors are associated with the zeta chain, a chain that it is key in mediating signaling via the TCR. The zeta chain is also a prominent tyrosine phosphorylated substrate in T cells. We therefore investigated the role of tyrosine kinases and zeta in T cells and NK cells. Our objectives were to understand better the role of PTK in the process of activation and to try to draw comparisons between signaling via the TCR and the FcR.

RESULTS AND PROGRESS

The role of tyrosine kinases in signaling in T cells and natural killer (NK) cells was investigated to determine their importance in signaling via the T-cell receptor and the NK Fc receptor. The prototypic tyrosine kinase gene v-src was expressed in the antigen-specific T-cell hybridoma, 2B4, using retroviral gene transfer. Approximately 20 separate clones were generated that expressed v-src. Because TCR expression is labile in the hybridoma cells, the clones were also screened for expression of the TCR and ~1/3 were found to express roughly normal levels of surface-expressed TCR as well as v-src. The presence of enzymatically active $pp60^{v\text{-}src}$ was confirmed by immunoprecipitation of metabolically labelled protein, in vitro kinase assay and analysis of tyrosine phosphorylated substrates in whole cell lysates by immunoblot analysis. We observed that cells that expressed v-src constitutively produced factor activity that supported the growth of the IL-2 dependent cell line, CTLL. This activity was inhibitable by anti-IL-2R antibody. IL-2 secretion was also inhibitable by forskolin and cyclosporin A. Studies are underway in collaboration with Dr. Gerald Crabtree at Stanford University to delineate the elements within the IL-2 gene that are responsible for the effect of v-src. Tentatively, this has been mapped to the NFkappa B site. Studies are therefore planned to determine if in vitro phosphorylation of the IkappaB and NFkappa B complex will influence the binding of NFkappa B to the appropriate elements of the IL-2 gene.

Among the substrates that were noted to be constitutively phosphorylated in v-src expressing cells was the zeta chain of the TCR. This provided a model to test several questions related to the importance of zeta as a substrate in TCR-mediated signaling. While there is now abundant evidence that zeta is a key protein in mediating the coupling of the TCR to the signal-transducing

machinery, the role of zeta as a substrate is still somewhat of a mystery. The hypothesis has been previously raised that tyrosine phosphorylation of zeta serves to uncouple the TCR from phosphoinositide hydrolysis. This was derived from studies in the autoimmune strains of mice, gld and lpr. In v-src expressing cells however, perturbation of the TCR anti-receptor antibody results in normal PI turnover and normal phosphorylation of TCRγ, a PKC substrate, IL-2 production and cell death. This suggests that tyrosine phosphorylation of zeta per se does not uncouple the TCR from signaling. However, a number of studies have indicated that perturbation of the TCR by antibody is by no means equivalent to the physiologic ligand. Studies are underway to directly compare antigen and antireceptor antibody as stimuli in v-src-expressing cells to test the hypothesis that tyrosine phosphorylation of zeta is a desensitizing signal. A second possibility was that zeta was a necessary substrate for the promotion of more distal events such as gene induction, independent of its role in coupling the receptor to kinases. To this end, subclones of v-src-expressing T cells were derived with varying levels of surface TCR and zeta. Expression of zeta did not correlate with IL-2 production, indicating that phosphozeta was not necessary for the induction of genes such as the IL-2 gene, in the presence of an active tyrosine kinase. As an extension of this project, T cells have been prepared that express the human EGF receptor as a second prototypic tyrosine kinase. These cells are currently being evaluated functionally but it is clear that the EGFR that is expressed is enzymatically active in these cells and that EGF treatment of T cells results in phosphorylation of a number of substrates. We are presently trying to determine if TCR-mediated signaling and EGFR-mediated signaling share common substrates.

Recently, it has become clear that NK cells, while lacking other constituents of the TCR, do express the zeta chain and it is part of the NK FcR complex. To determine if zeta could serve a potential coupling role for the FcR analogous to its role in T cells, we investigated FcR mediated phosphorylation of zeta in NK cells. Perturbation of the FcR with anti-receptor antibodies or with antibody-coated targets resulted in zeta chain phosphorylation. In contrast, IL-2, PMA and ionomycin, and NK targets all failed to induce zeta chain phosphorylation. These studies are consistent with the interpretation that the NK Fc receptor, like the T-cell receptor appears to be coupled to a tyrosine kinase as well as phospholipase C and phosphoinositide hydrolysis. As an extension of this study, we have begun to search for the kinase that is associated with zeta in NK cells. Preliminary evidence indicates that indeed kinase activity appears to be specifically co-precipitated with zeta in NK cells. At present we are attempting to determine if this kinase is a novel protein or is a previously described kinase.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09283-06 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Control of Human Interferon-γ 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

COOPERATING UNITS *(if any)*
Biological Carcinogensis and Development Program, Program Resources, Inc., NCI-FCRDC (V. Ciccarone, M. Beckwith, L. Dorman, C. Moratz).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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 previously identified regions of human interferon-γ (IFN-γ) genomic DNA which can enhance gene expression in murine and human 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, and is stimulated by PMA in T cells. These results indicate that control of IFN-γ 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. This hypothesis is supported by the findings that interleukin-2 directly induces IFN mRNA in large granular lymphocytes but has no effect on IFN transcription in resting T cells, thus indicating that extra cellular signaling of gene expression is distinct in different lymphocyte subsets.

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

OBJECTIVES

We have chosen 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 IFN-γ 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. These results indicate that 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 continued to dissect 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 both enhancer and repressor activity can be detected within the 300 bp 5′ to the TATAA box. This enhancer activity demonstrates increased activity after PMA stimulation in either the murine T-cell line or human peripheral blood T cells but appears to be inactive in a murine fibroblast cell line and human B-cell lines. Additional enhancer activity was also localized to a 250 bp region of the first intron and this enhancer activity was also increased by PMA in the murine T cell line or human T cells and was expressed constitutively in the murine fibroblast cell line and human B-cell lines.

We have also identified a unique cell line which appears to be altered in the control of IFN-γ gene expression. This human cell line expresses constituitive IFN-γ and expression is induced 2 fold by PMA, 4-6 fold by IL-2 and 8-12 fold by IL-1 + IL-2. Most interestingly, cyclosporin A, a potent inhibitor of IFN gene expression, significantly enhances IFN-γ production when added in combination with the above mentioned agents. The mechanisms responsible for this altered induction are under investigation.

The current studies presented here concerning the regulation of IFN-γ gene expression offer an opportunity to dissect the role of a specific immunoregulatory 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

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09303-04 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

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:	Y. Pang	Fogarty Fellow	LEI, NCI

COOPERATING UNITS *(if any)*
Biological Carcinogensis and Development Program, Program Resources, Inc., NCI-FCRDC (R. R. S. Kantor, S. Giardina); DM, DCT, NCI (K. Cowan, 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-FCRDC, Frederick, Maryland 21702-1201

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 π (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.

In addition to the above studies, 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, ovarian, and myeloma tumor cell lines resistant to chemotherapeutic drugs have been developed by in vivo passage in nude mice or in vitro culturing. These cell lines are being utilized to analyze the effects of biological response modifiers (e.g. IL-6) on the drug resistance phenotype and growth characteristics of these cells.

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Yubin Pang	Fogarty Fellow	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 marker for cervical cancer, 2) to study the mechanisms of tumor cell resistance to chemotherapeutic drug and develop methods to overcome this resistance, and 3) to investigate the role of IL-6 in the development of human multiple myeloma.

RESULTS

Our previous immunohistological studies have shown that GST-π is a potential marker for cervical cancer as normal cervical epithelium does not express GST-π but >90% of cervical dysplasias and carcinoma express GST-π. We have now demonstrated an excellent correlation between cervical dysplasias that express GST-π and those that harbor DNA for cervical cancer-associated human papilloma viruses 16/18 and 31/33/35. Additionally, GST-π immunostaining co-localized with HPV as detected by in situ hybridization. No correlation was found between other HPV types (6/11) and GST-π immunostaining. Collaborative clinical studies will analyze GST-π and HPV in Pap smears and determine if either of these factors are useful to predict disease progression.

Multidrug resistance is a major cause for cancer chemotherapy failure. We have established a model of multidrug resistance in which the human colon carcinoma cell line Ht-29 was transfected with the MDR-1 gene and passaged in an ascites form in nude mice. We are currently evaluating the effects of agents known to reverse this form of drug-resistance (e.g. veramil, cyclosporin and antibodies to the p170 protein) in this model system.

Studies of freshly isolated human multiple myeloma cells have shown that they proliferate in response to IL-6 and conversely that antibodies to IL-6 inhibit their proliferation. This and other observations have suggested a role for this lymphokine in the development of human multiple myeloma. To evaluate if the disruption of IL-6-mediated growth stimulation of multiple myeloma may be a useful therapeutic approach in this disease we are currently producing neutralizing monoclonal antibodies to IL-6 and the p-80 IL-6 receptor. These antibodies will be tested for their ability to inhibit myeloma growth in vitro and in a mouse model system. IL-6 receptor antibodies will also be useful in the biochemical characterization of the p-80 receptor and associated proteins. Studies to define IL-6 receptor signal transduction mechanisms at the biochemical level are also ongoing.

PUBLICATIONS - None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09326-02 LEI

PERIOD COVERED

October 1, 1989 through September 30, 1990

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:	D. Reynolds	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*

Biological Carcinogenesis and Development Program, Program Resources Inc., NCI-FCRDC (L.A. Eader); Experimental Therapeutics Section, LEI (R.H. Wiltrout, H. Futami).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	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.)*

We have investigated the mechanism by which flavone-8-acetic acid (FAA) acts to enhance immune responsiveness in murine tumor model systems. Analysis of spleen mRNA samples after FAA administration indicates that both interferon (IFN) α and tumor necrosis factor (TNF) mRNA can be detected within 1-3 hours. Further studies have indicated that flavone can act directly on mouse spleen cells in vitro. These results indicate that FAA is a potent biological response modifier which acts by directly stimulating gene expression of important immunoregulatory proteins.

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, 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. One possible mechanism for these effects is through the induction of cytokines. We have previously 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 and in vitro, of mRNA for IFN or other cytokines following FAA administration.

ACCOMPLISHMENTS

A panel of cytokine probes was employed in the in vitro screening of spleen cells RNA and the temporal expression of induced genes was examined. IFN (Type 1 and 2) mRNA in the spleen cells was upregulated within 3.0 h after FAA, while an upregulation of TNF mRNA was detected by 1 hr. after in vitro treatment. A dose dependent upregulation by FAA of cytokine mRNA was also observed. No induction of interleukin 2 mRNA was observed in the spleen cells in these studies. Fractionation of the spleen cells has indicated that the T cells were responsible for the IFN-γ mRNA, all subsets expressed TNF mRNA and that the monocytes and B cells may produce IFN-α mRNA in response to FAA. These results demonstrate that FAA can act as a potent inducer of cytokine mRNA in vitro and suggest that the immunomodulatory and immunotherapeutic effects of FAA may be a direct result of FAA stimulation of cytokine gene expression.

Additional studies are being undertaken to determine if FAA can also induce cytokine gene expression in human lymphocytes in vitro.

Our increased understanding of how single agents (such as FAA) or combinations of agents (FAA + IL-2) effect cytokine gene expression would permit 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. Identification of the biochemical events which trigger cytokine gene induction by FAA would also permit the development of more therapeutically potent FAA drug analogs.

PUBLICATIONS

Futami H, Pilaro AM, Gruys ME, Back TT, Young HA, Wiltrout RH. In vivo distribution and cytokine gene expression by enriched mouse LAK effector cells, Biotherapy 1990;in press.

Mace KF, Hornung RL, Wiltrout RH, Young HA. Correlation of in vivo cytokine gene expression by flavone acetic acid with strict dose dependency and therapeutic efficacy against murine renal cancer, Cancer Res 1990;50:1742-7.

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Wiltrout RH, Hornung RL, Futami H, Young HA, Sayers TJ. Involvement of cytokines in combined modality preclinical approaches to cancer treatment. In: Rees RC ed. The biology and clinical applications of interleukin. United Kingdon: Oxford Univ Press, 1990;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09345-01 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Studies of Cellular Cytotoxicity

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

PI:	H. A. Young	Head	LEI, NCI
Others:	M. Smyth	Guest Researcher	LEI, NCI
	Y. Norihisa	Fogarty Fellow	LEI, NCI

COOPERATING UNITS *(if any)*
National Research Council, Canada (S. Anderson); Univ. of Toronto (J. Roder).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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.)*

We have been studying gene expression in cytotoxic immune cells with specific emphasis on large granular lymphocytes (LGLs) and cytotoxic T cells. We have molecularly cloned a new gene which appears to be a receptor for LGLs and may also be expressed in T cells. We have also correlated cytotoxic function with expression of a cytotoxic protein, perforin, and have identified extracellular signals which upregulate perforin mRNA expression. These studies begin to define, at the molecular level, the genes involved in the development and maturation of cytotoxic immune effector cells.

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Mark Smyth	Guest Researcher	CMIS, LEI, NCI
Yoko Norihisa	Fogarty Fellow	CMIS, LEI, NCI

OBJECTIVES

This project is designed to investigate the molecular events which occur upon activation of immune cytotoxic effector mechanisms. Using cDNA probes, we are identifying those genes which are expressed in cytotoxic T cells and LGLs and which appear to play an important role in the ability of these cells to kill tumor cells.

ACCOMPLISHMENTS

This laboratory, in collaboration with Dr. John Ortaldo, has isolated a unique gene which functions as a receptor on natural killer cells (LGLs) for attachment to their tumor target. The gene is located on chromosome 3, codes for a mRNA of 7-8kb and is expressed only in T cells and LGLs. The human cDNA was also found to have homology to genes in all vertebrate species analyzed thus far. The gene contains a unique structure in the 5' extracellular region which suggests that it may have a cis-trans isomerase activity. Currently studies are focused on defining conditions which alter mRNA expression and on the effects of introducing cDNA expression vectors containing portions of the cDNA into appropriate lymphocyte cell lines in order to evaluate structure-function relationships.

We have also identified a gene, perforin, which shows increased mRNA expression in resting human peripheral blood T cells upon treatment with high dose IL-2. Further analysis of the T-cell population has shown that the perforin gene is preferentially induced in CD-8+ T cells but is constituitively expressed and not inducible in LGLs. We have also found that the levels of IL-2 needed to induce perforin gene expression can be lowered by up to two logs when IL-6 is added to the culture medium. Additional studies have indicated that the expression of the perforin mRNA correlates closely with the cytotoxic activity of the cells.

Further studies will be focused on upregulating immune system functions, including the enhancement of natural killer cell and cytotoxic T-cell activity, by activation of the cell surface expression of our newly identified receptor gene and correlating this activation with perforin gene expression. Thus based on a precise understanding of NK receptor gene expression, it may be possible to design unique drugs which provide a general stimulus to the immune system. Furthermore, based on our knowledge of how this receptor gene is controlled, it may be possible to create new genes whose expression is restricted to immune cells thus providing a unique form of therapy not currently available.

PUBLICATIONS

Gotlieb WH, Takacs L, Young HA, Gusella L, Durum SK. Growth of immature (double negative) rat thymocytes in the presence of IL-1, J Immunol 1990;144:2072-81.

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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, Switzerland: Karger, 1989;22-7.

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Smyth MJ, Ortaldo JR, Shinkai Y-I, Yagita H, Okumura K, Young HA. Interleukin-2 induction of pore-forming protein gene expression in human peripheral blood $CD8^+$ T cells, J Exp Med 1990;171:1269-81.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09262-08 LEI

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antitumor Effects of BRM-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	Staff Fellow	LEI, NCI
	H. Futami	Nakasone Fellow	LEI, NCI
	M. E. Gruys	Biologist	LEI, NCI
	T. A. Gregorio	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*
CMIS, BRMP (H. Young); Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (T. Sayers, R. Hornung, L. Eader, K. Komschlies-McConville).

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	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.)*

The investigational drug Flavone acetic acid (FAA) upregulates the genes for IFNs γ and α as well as TNF-α, and synergizes with rIL-2 for the treatment of murine renal cancer (Renca) and the C26 murine colon carcinoma. Subsequently, IFN-α and IFN-γ were shown to synergistically inhibit the proliferation of Renca in vitro and to synergize with IL-2 for treatment of localized Renca in vivo in euthymic but not athymic mice. Since CD8+ T cells are critical for the antitumor effects of FAA + rIL-2, current studies are focused on whether recognition of Renca-associated tumor antigen(s) occurs by T cells utilizing a select subset of T-cell receptor v-region genes. Alkalinization of Renca-bearing mice, in a manner analogous to that used for clinical trials of FAA, inhibited the ability of FAA to induce cytokine genes and proteins, as well as its therapeutic synergy with rIL-2. FAA induces cytokine genes directly in vitro concentrations of FAA ≥ 100 μg/ml preferentially induce the gene for IFN-γ in CD8+ T cells, while the gene for IFN-α is preferentially induced in B cells. The TNF-α gene is induced in all subsets studied. Induction of gene expression was followed by the detection of IFN activity in the supernatants of treated splenic leukocytes. Since the production of cytokines contributes to the augmentation of cytotoxic effector cell activity, we have also studied the ability of FAA to augment NK and LAK activities in vitro. Pretreatment of mouse splenic leukocytes with 500 μg/ml FAA alone induces some NK activity (12 LU30), while suboptimal (10 U/ml) and optimal (1000 U/ml) IL-2 yield 45 and 260 LU30 of NK activity, respectively. Pre-treatment with 100 μg/ml or 500 μg/ml FAA enhances the ability of IL-2 to augment NK activity by 2-3 fold and enhances IL-2-induced LAK generation by approximately 2 fold. These results demonstrate that FAA can act directly as an immunomodulator, and suggest that in vitro models may be useful to elucidate its mechanism of action at the molecular level. Finally an apparently novel tumor cytostatic factor has been identified in lysates of rat RNK tumor lines and is being biochemically characterized prior to molecular cloning.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Anne M. Pilaro	Staff Fellow	ETS, LEI, NCI
Hitoyasu Futami	Nakasone Fellow	ETS, LEI, NCI
M. Eilene Gruys	Biologist	ETS, LEI, NCI
Theresa Gregorio	Biologist	ETS, LEI, NCI

OBJECTIVES

The overall objective of this project is to develop preclinical strategies by which immunoactive biological response modifiers (BRMs) and recombinant cytokines can be used in concert for the treatment of cancer in mice. Additional studies are designed to determine the cellular and molecular mechanism(s) by which 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 cancers, and the basis for induction of specific antitumor immunity during this process.

2. To determine the role of selected cytokines (TNF-α, IFN-α, γ, IL-6, and IL-7) in the treatment of various mouse tumors by LAK + IL-2 and flavones + IL-2.

3. To identify and characterize novel leukocyte-derived antitumor factors.

RESULTS

There are three major areas of study in progress. First, a major focus of the research is on the development and utilization of IL-2-based combination approaches to cancer treatment in mice. Specific approaches developed include: 1) studying the mechanisms by which flavones plus rIL-2 mediate antitumor effects against Renca; 2) studying the ability of cytokines induced by flavones, including FAA, to mediate antitumor effects in combination with rIL-2 against the Renca and C26 tumors; and 3) studies are in progress to biochemically characterize and molecularly clone a newly identified factor that is cytostatic for tumor cells in vitro.

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. 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, in vitro exposure of leukocytes to FAA upregulates the expression of mRNA for TNF-α in CD4+ and CD8+ T lymphocytes, in B cells, and preferentially induces the genes for IFN-α and IFN-γ in B cells and CD8+ T cells, respectively. Further, active proteins for

IFN and TNF can be detected in the serum of FAA-treated mice. Subsequent studies performed in collaboration with Drs. Thomas Sayers and Ronald Hornung (PRI) suggest that IFN-α + IFN-γ can have direct antiproliferative effects against Renca in vitro and against localized intraperitoneal Renca in euthymic but not athymic mice. A role for FAA induced cytokines is further 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 IFN-α, IFN-γ, and rIL-2 were also immune to rechallenge. The critical subset stimulated by FAA + IL-2 for the anti-Renca response is the CD8+ T lymphocyte. Depletion of this subset in vivo by repeated administration of anti-Ly2.2 antibodies abrogates the therapeutic efficacy of FAA + rIL-2. T cells isolated from various lymphoid tissues and tumors, during and following FAA and IL-2 treatment of Renca bearing mice, are being studied for specific proliferation and cytotoxicity against Renca. In collaboration with Dr. Kristin Komschlies-McConville (PRI), MoAb to various TcR chains and their V-regions are being used to examine whether these cells bear a selected subset of the TcR repertoire. Moreover, we wish to determine whether T cells that respond to tumor cells have altered functions in other assays such as mitogen and anti-CD3 stimulation. We will also determine whether any observed selection of the TcR repertoire occurs at the local versus systemic level, and will investigate the role the thymus plays in this selection.

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 TNF-α, IFN-γ, and IFN-α. These effects can be overcome by administration of higher doses of FAA.

In additional studies, we have also demonstrated that extracts of the granules obtained from a rat NK (RNK) tumor contain a constituent(s) which is growth inhibitory to a variety of tumor cells in vitro. The effect seems to be predominantly cytostatic rather than cytotoxic. Furthermore, distinct morphological changes occur in sensitive cells in addition to the growth inhibition. The observed biological activity does not appear to be due to TNF-α, TGF-β, or any other known cytokine tested to date. Using biochemical fractionation procedures, we have partially purified the cytostatic molecule which appears to be a protein of about 32 KDa in both reduced and non-reduced form. It is a heparin-binding protein that is denatured by heat treatment, but is relatively resistant to trypsin. Currently we are trying to purify the molecule to homogeneity utilizing a variety of procedures including sizing columns, ion-exchange, heparin affinity, and hydrophobic interaction chromatography. We anticipate being able to soon perform an N-terminal amino acid sequence analysis on the molecule to determine whether it is a novel structure or not. If novel we will attempt molecular cloning of the molecule.

PUBLICATIONS

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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;49:3562-7.

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Sayers TJ, Wiltrout RH. Regulation and function of natural killer cells. In: Rogers TJ, Gilman S, eds. Immunopathology. Caldwell, NJ: The Telford Press, 1989;13:343-79.

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Pearson JW, Hedrick E, Fogler WE, Bull RL, Ferris DK, Riggs CW, Wiltrout RH, Sivam G, Morgan AC, Groves E, Longo DL. Enhanced therapeutic efficacy against an ovarian tumor xenograft of immunotoxins used in conjunction with recombinant interferon-α. Cancer Res 1990;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09288-05 LEI

PERIOD COVERED

October 1, 1989 through September 30, 1990

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
	W. E. Fogler	Senior Investigator	CTEP, NCI

COOPERATING UNITS *(if any)*

Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (T. Sayers and R. Kantor); Cetus Corp. (E. Groves); NeoRx Corp. (C. Morgan)

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	1.50	.75

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 antitumor effects of two immunotoxins (IT) were evaluated in vitro and in vivo against the human ovarian carcinoma cell line, OVCAR-3. The ITs used were an anti-carcinoma MoAb coupled to Pseudomonas exotoxin (NR-LU-10/PE) and a IT composed of the recombinant A chain of ricin covalently attached to a MoAb directed to the transferrin receptor (454A12/rRTA). Protein synthesis was inhibited in a dose-dependent manner in OVCAR-3 cells incubated in vitro with either NR-LU-10/PE or 454A12/rRTA (IC50=1 and 75 ng/ml) respectively. Concomitant incubation in vitro of OVCAR-3 cells with NR-LU-10/PE or 454A12/rRTA and a non-cytotoxic concentration of rhIFN-α was shown to potentiate the inhibitory activity of the ITs via a mechanism distinct from antigenic regulation. Studies showed that the MST of mice injected i.p. with 4,000,000 OVCAR-3 cells was 46 days. Cohorts of mice that received intracavitary treatment beginning five days post-tumor cell injection with either 0.25 or 0.5 μgs of NR-LU-10/PE every other day for a total of ten treatments exhibited significantly increased MSTs of 63.0 and 104 days respectively (P=0.0001). Likewise, the i.p. injection of either 2.5 or 10 μgs of 454A12/rRTA administered by an identical regimen, resulted in MSTs of 89.0 and >120.0 days, respectively (P=0.0001). When rhIFN-α was administered i.p. in conjunction with these doses of either IT, a significant increase in MST was observed in comparison to mice given IT alone. The combination of 50,000 U rhIFN-α and 0.25 μgs NR-LU-10/PE resulted in 67% long-term survivors compared to only 13% survival of mice given IT alone. Similarly, 2.5 μgs of 454A12/rRTA plus rhIFN-α resulted in 89% long-term survivors compared to 454A12/rRTA alone (29%). Preliminary studies have revealed that a human colon line expressing the MDR gene, Ht-29(R), is highly resistant to multiple i.p. treatments of vincristine (2mg/kg). Investigations are underway to attempt to reverse this resistance in vivo utilizing various drugs and biological agents.

PHS 6040 (Rev. 1/84) GPO 914-911

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
William E. Fogler	Senior Investigator	CTEP, NCI

OBJECTIVES

The aim of this project is two-fold: (1) To evaluate the intracavitary administration of immunotoxins (IT) alone or in conjunction with potentiating agents or drugs against a human ovarian tumor confined to the peritoneal cavity of athymic mice. The specific objectives of this aim are as follows: (a) to define optimal treatment protocols, i.e. dose and regimen to maximize the therapeutic effect of specific ITs against localized tumor growth and (b) to assess the feasibility of combination therapy, i.e. BRMs or chemotherapy in combination with ITs to further enhance the therapeutic efficacy of ITs against peritoneal disease and (2) To develop models to overcome drug resistance in human tumors in vivo. The specific aims are to examine various drugs or the use of specific monoclonal antibodies (MoAb) coupled to cytotoxic substances to attempt treatment of drug resistant tumors.

RESULTS

1. In Vitro Activity of ITs Against OVCAR-3.

The incorporation of [^{3}H] leucine was used to assess the in vitro activity of NR-LU-10/PE and 454A12/rRTA. Investigations revealed that protein synthesis was inhibited in a dose-dependent manner in OVCAR-3 cells incubated in vitro with NR-LU-10/PE. The IC_{50} of NR-LU-10/PE was 0.5-1.0 ng/ml with maximal activity obtained at 4 ng/ml NR-LU-10/PE (89% inhibition). The growth inhibition obtained with 4 ng/ml NR-LU-10/PE was completely prevented by an antigen-saturating amount (50 μg) of the unconjugated, NR-LU-10 (89% vs. 22%). Under identical culture conditions, incubation of OVCAR-3 cells with an irrelevant IT, anti-TAC/PE, did not result in appreciable levels of protein synthesis inhibition (range of 2-17% inhibition). OVCAR-3 cells treated with 454A12/rRTA similarly exhibited a dose-dependent inhibition of [^{3}H] leucine incorporation. Maximal activity (80% inhibition) was obtained at 400 ng/ml with an IC_{50} of 75-100 ng/ml for 454A12/rRTA. Incubation of OVCAR-3 with 400 ng/ml 454A12/rRTA and 250-fold excess (100 μg) unconjugated 454A12 significantly decreased the maximal activity of the IT (80% vs. 18% inhibition). Protein synthesis was unaffected following incubation of OVCAR-3 cells with a similar concentration of an irrelevant IT, MOPC/rRTA.

We further investigated whether the inhibition of protein synthesis observed with the ITs could be modulated by recombinant human interferon-α (rhIFN-α). Concomitant incubation of OVCAR-3 cells with NR-LU-10/PE (0.5 ng/ml) and $IC_{0,5}$, or $_{15}$ levels of rhIFN-α was observed to be 47, 76, and 78% inhibition compared to 24% inhibition of cells incubated with NR-LU-10/PE alone. Inhibition of protein synthesis of OVCAR cells treated with 80 ng/ml 454A12/rRTA alone was observed to be 34%. When this concentration of IT was used in combination with $IC_{0,5}$, or $_{15}$ levels of rhIFN-α the inhibition was found to be 52, 70, and 80%, respectively.

2. In Vivo Antitumor Activity of ITs Against OVCAR-3

In vivo studies have been performed using a human ovarian carcinoma (OVCAR-3) in athymic mice. Following i.p. injection of four million cells, our investigations 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. To assess the in vivo antitumor efficacy of NR-LU-10/PE, nude mice were injected i.p. with four million OVCAR-3 cells. Five days after tumor cell injection, groups of mice were treated i.p. every other day for a total of 10 treatments with saline or either 0.5 or 0.25 μg of NR-LU-10/PE. At the initiation of therapy (day 5) approximately 1×10^7 OVCAR-3 ascites cells could be recovered from the peritoneal cavities of individual mice. The median survival time (MST) of mice treated with saline alone was 46.0 days. In contrast, mice treated i.p. with either 0.5 or 0.25 μg of NR-LU-10/PE showed significant increases ($P = 0.001$) in MST (104 and 63 days, respectively), when compared to the control group. The in vivo antitumor activity of NR-LU-10/PE was further enhanced by co-administration of rhIFN-α. Treatment of tumor-bearing mice with 0.25 μg of IT in combination with 5×10^4 U rhIFN-α resulted both in an increase in the MST (> 120 days, $P = 0.0005$) and, in comparison to NR-LU-10/PE alone, an increased percentage of long term survivors when the study was terminated at 120 days. Likewise, 71% long-term survivors were obtained in the group of mice that received 0.5 μg of NR-LU-10/PE and 5×10^4 U rhIFN-α as compared to 40% survivors with IT alone. The administration of 5×10^4 U rhIFN-α alone had no therapeutic effect against OVCAR-3. Similar results were obtained following treatment of OVCAR-3-bearing mice treated with 454A12/rRTA alone or in combination with rhIFN-α. The i.p. administration of either 2.5 or 10 μg of the IT alone resulted in MSTs of 89.0 and > 120 days respectively $P = 0.001$, when compared to the control group. Concomitant treatment of mice with 5×10^4 U rhIFN-α and 2.5 μg of 454A12/rRTA resulted in 89% long-term survivors as compared to 29% survivors in the group of mice that received the same dose of IT alone. No significant differences were obtained in MST for groups of mice that received 10 ug of 454A12/rRTA either alone or in combination with rhIFN-α. However, the combined therapy resulted in 88% long-term survivors as compared to 65% survival in mice that received IT alone. The use of antigen-irrelevant ITs did not result in an increase of MST as compared to saline treated controls. Subsequent studies revealed the recurrent population of ascites cells were equally or more sensitive to their relevant IT than tumor cells obtained from untreated mice. Therefore, the data suggest that the recurrence of OVCAR-3 cells was not due to the selection of IT-resistant or target antigen negative tumor cell variants. To examine if rhIFN-α influenced expression of NR-LU-10 or 454A12 binding epitopes on OVCAR-3 cells, flow cytometric analysis and competiive inhibition studies were performed. The

results indicated that rhIFN-α did not substantially increase expression of either the NR-LU-10 or 454A12 antigen. Studies, are currently underway to evaluate the combination of rhIFN-α and the two ITs against the ovarian tumor when administered against a greater tumor burden, i.e. 10 and 15 days post-tumor cell inoculation. Finally, investigations are ongoing to evaluate the therapeutic efficacy of the ITs alone or in combination with rhIFN-α following cytoreductive therapy in this case utilizing cytoxan and cis-platinum as debulking agents.

3. Development of Models to Overcome Drug Resistance in Human Tumors In Vivo.

It has been reported that a gene responsible for multi-drug resistance, termed MDR, encodes a drug-transport protein referred to as P170 or P-glycoprotein that acts as a pump to transport drugs out of the cell. This resistance involves a specific group of naturally occurring drugs such as adriamycin, vincristine, vinblastine, ACT D and VP-16. Recently, studies were carried out in vivo to evaluate the antitumor effect of vincristine against a vincristine-sensitive human colon line, Ht-29(S) and a vincristine resistant colon line, Ht-29(R), created by gene transfection. Mice were inoculated i.p. with either 3 x 10^6 Ht-29(S) or Ht-29(R) ascites cells on day zero. Beginning 10 days post-tumor cell inoculation, and then weekly for two weeks for a total of three treatments, mice were treated i.p. with 2 mg/kg of vincristine. Preliminary data indicate that vincristine significantly increased (P=.001) the MST from 31.0 days in the control group to 86.0 days in the group of mice that received vincristine. In contrast, mice bearing the i.p. Ht-29(R) tumor following treatment with a similar regimen of vincristine exhibited at MST of 43.0 days. With the recent development of a MoAb (MRK-16) that reacts with a portion of the P-glycoprotein that is present on the surface of drug resistant cells, studies are underway in vivo to explore whether the MRK-16 MoAb is capable of neutralizing the function of the MDR gene present on the surface of the Ht-29(R) colon tumor. Future studies will entail the use of anti-P-glycoprotein-toxin conjugate against the Ht-29(R) tumor line in nude mice. Finally, studies will be undertaken in vivo utilizing such compounds as verapamil, cyclosporin A, and quinidine against the vincristine resistant colon tumor line. These compounds allow cytotoxic chemicals to accumulate within resistant cells, thus overcoming multiple-drug resistance.

PUBLICATIONS

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;49:4990-5.

Pearson JW, Hedrick E, Fogler WE, Bull RL, Ferris DK, Riggs CW, Wiltrout RH, Sivam G, Morgan AC, Groves E, Longo DL. Enhanced therapeutic efficacy against an ovarian tumor xenograft of immunotoxins used in conjunction with recombinant interferonα. Cancer Res 1990;50:6379-88.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09322-02 LEI

PERIOD COVERED
October 1, 1990 through September 30, 1991

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:	A. M. Pilaro	Staff Fellow	LEI, NCI
Others:	R. H. Wiltrout	Head	LEI, NCI
	M. J. MacPhee	Visiting Fellow	LEI, NCI
	P. L. Echeagaray	Chemist	LEI, NCI
	H. M. Williams	Stay-in-School	LEI, NCI

COOPERATING UNITS *(if any)*
Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (T. J. Sayers); UMDNJ, Robert Woods Johnson Med. School (F. M. Robertson)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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 increases in both the number and cytolytic activity of large granular lymphocytes (LGL) in 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. Thus, cytokines or other mediators released in these organs following BRM treatment may be responsible for the recruitment and/or activation of LGL observed. The mechanism by which these cells accumulate is being studied in vitro, using a modified Boyden chamber chemotaxis assay and collagen-coated, millipore filters. Using this technique, we have examined the release of chemotactic activity for LGL from liver cell cultures treated in vitro with BRM. Although the BRM themselves did not stimulate LGL migration, treatment of isolated cells induced the production and release of soluble factors that were chemotactic for LGL in vitro. Since these BRM are known to induce the release of a number of cytokines from immune cells which may be important in recruiting LGL in vivo, we have studied the chemotactic activity of these agents for LGL in vitro. We have determined that several different cytokines can induce chemotaxis of LGL, including partially purified rat interferon α/β (IFN-α/β), recombinant human (rh) interleukin 2 (IL-2), rhIL-6, and rh tumor necrosis factorα (TNFα). These cytokines stimulated chemotaxis of LGL in a dose and time-dependent manner, suggesting that local release of these agents may contribute to the recruitment of LGL and other leukocytes in vivo. Future studies are focused on the characterization and identification of the chemotactic factors released from isolated liver cells. Using the peritoneal cavity as a model, we have also begun to study the mechanisms by which other leukocyte types recognize and respond to stimuli in vivo. These studies will allow us to determine the role of cytokines in the recruitment of different populations of leukocytes during an inflammatory response.

PROJECT DESCRIPTION

PERSONNEL

Anne M. Pilaro	Staff Fellow	ETS, LEI, NCI
Robert H. Wiltrout	Head	ETS, LEI, NCI
Martin J. MacPhee	Visiting Fellow	ETS, LEI, NCI
Patricia L. Echeagaray	Chemist	ETS, LEI, NCI
Hope M. Williams	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 characterize and identify LGL chemotactic factors released from BRM-treated liver cells in vitro.

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 following treatment with BRMs.

RESULTS

Because NK cells have been demonstrated to accumulate in the liver after administration of BRM, we examined the release of chemotactic factors from isolated liver cell cultures following treatment with various BRM. Our initial studies were conducted in rats, since they provide a convenient way of obtaining large numbers of highly enriched LGL, hepatocytes and liver macrophages (Kupffer cells). Liver cells were isolated from F344 rats by collagenase perfusions, and enriched for hepatocytes or Kupffer cells by centrifugation on either Percoll or metrizamide gradients, respectively. Isolated cells were cultured for 24 hr to re-establish membrane integrity, then treated for 4 hr with various concentrations of BRM, including LPS, C. parvum, acetaminophen, MVE-2, or the IFN inducer poly ICLC. Supernatants from BRM-treated cells were then tested 24 hr later for induction of LGL chemotaxis. Using the modified Boyden chamber technique and collagen-coated millipore filters, rat LGL were found to migrate towards

supernatants from liver cells treated with a number of different BRM. Treatment of hepatocytes with either acetaminophen, C. parvum or LPS in vitro resulted in the appearance of LGL chemotactic activity in supernatant samples, while MVE-2 or ICLC treatment had no effect on chemotactic factor production. Interestingly, acetaminophen, MVE-2 or C. parvum treatment of liver macrophages did not induce chemotactic factor release, while LPS or poly ICLC treatment resulted in 2-3 fold increases in activity over background levels. These effects were dose-related, and corresponded to levels of BRM that would be achievable in vivo. Furthermore, none of the BRM themselves induced LGL migration, confirming that the migration observed in vitro in supernatant samples is due to release of specific factors that stimulate LGL chemotaxis, and suggesting that induction of chemotactic factors may contribute to the mechanism by which these agents recruit LGL in vivo. Future studies will focus on characterizing and identifying these chemotactic factors to determine whether they are novel molecules, or represent a new function for previously described mediators.

Since poly ICLC and LPS are both known to induce the release of IFNs and other biologically active cytokines from leukocytes or hepatocytes, we next tested the ability of several recombinant human (hr) cytokines to induce LGL chemotaxis. When 1000 U/ml of hrIL-1α, IL-4 or IL-8 or rat rIFN-γ were placed in the lower wells of the Boyden chamber, no stimulation of migration above background levels occurred. However, chemotaxis of LGL was stimulated in a dose-dependent fashion (2-4 fold) when hrIL-2, IL-6, TNFα or partially purified rat IFN-α/β were placed in the lower wells. These results support our hypothesis that cytokines released by BRM-treated cells could mediate, at least in part, the in vivo recruitment of LGL. To study this point, future experiments are planned to examine the production of these cytokines by BRM-treated liver cells. Initially, in collaboration with Dr. Fredika Robertson at the Robert Woods Johnson Medical School, we will utilize flow cytometric techniques designed to blot pure populations of immune cells directly onto nitrocellulose filters. Messenger RNA for specific cytokines can then be analyzed by conventional techniques. The advantage to these methods is that very few cells are required as compared to Northern blot analysis, and multiple samples may be collected and analyzed at any one time. Studies are in progress using mouse liver cells to extend and confirm these observations. Additional studies, utilizing neutralizing antibodies directed against specific cytokines are also planned as a method to characterize cytokines present in BRM-treated liver cell supernatants, and to examine their contribution to the induction of LGL chemotaxis.

Another potential mechanism by which NK cells may be recruited in vivo is by the release of acute phase reactant proteins following BRM treatment. These proteins have been demonstrated to be released from hepatocytes following exposure to activated macrophages or cytokines such as IL-6. In rats, the major acute phase reactant protein released following stimulation is C-reactive protein (CRP). CRP has been demonstrated to have activating effects on several types of immune cells as well as to induce chemotaxis of peripheral blood monocytes. To examine whether CRP may be an active mediator in the recruitment of LGL to the liver, we tested a synthetic fragment of the biologically active portion of CRP as well as the native molecule in the Boyden chamber assay. Both agents were found to stimulate migration of LGL in a dose-dependent manner, suggesting that release of this molecule in vivo may also be important in the

infiltration of LGL into tissue sites. Our future plans involve treating liver cell supernatants with neutralizing antibodies to CRP, as well as to other cytokines, to examine their involvement in mediating LGL recruitment.

To extend these studies to examine the effects of cytokines in the in vivo recruitment of LGL, in collaboration with Dr. Thomas Sayers (BCDP, PRI) 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 NK 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 the in vitro data obtained for induction of LGL chemotaxis to TNFα, mouse NK cells did not accumulate in the peritoneum in response to local injection of this cytokine. However IFN-γ or IL-2 injection resulted in increases in levels of peritoneal NK. Augmentation of peritoneal NK by IL-2 could be significantly inhibited by neutralizing monoclonal antibodies to IFN-γ, suggesting that in situ generation of IFN-γ played a role in responses to IL-2. Treatment of peritoneal cells with IFN-γ in vitro resulted in neither increases in NK lytic activity nor increases in LGL-1+ cells or BLT esterase activity. These results suggest that NK cells enter 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 IFN-γ 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.

Migratory leukocytes must be able to bind to and penetrate capillary endothelial cells and their underlying basement membrane before extravasation can occur. Additionally, therapeutic regimens with IL-2 and LAK effector cells, or with combinations of cytokines in humans have shown a dose-limiting toxicity referred to as vascular leak syndrome. Evidence exists that this syndrome involves interactions between circulating effector cells and vascular endothelium. These and other interactions of NK or LAK cells with capillary endothelium have not been well characterized, primarily because the effector cells that have been most extensively studied are of murine origin, while studies utilizing cultured endothelial cells have almost exclusively been conducted with cells of human origin. This mismatch of available cells has largely been due to the difficulties in purifying and subsequently studying human effector cells in vivo, and to obtaining and culturing large numbers of sufficiently pure murine endothelial cells in vitro.

To address these problems, we have developed techniques to generate isolated vascular beds in vivo employing collagen sponges containing angiogenic endothelial cell growth factors. Implantation of these sponges in mice allows us to generate vascular beds in vivo that originate from various organ systems, but

are devoid of parenchymal cells. Following collagenase digestion of the sponges, we have obtained recoveries of up to 1 x 10^6 endothelial cells/mouse. Several different strains of mice were tested for the ability to respond and generate vascularized sponges by this technique, with the DBA/2 strain yielding a much more profound response than other strains tested. The recovered endothelial cells were induced to proliferate in vitro using combinations of growth factors and feeder cells, and were assayed for purity utilizing fluorescently labeled acetylated low density lipoproteins or the endothelial cell-specific monoclonal antibody MECA-32 and flow cytometry. Initial purity of the isolated cells was approximately 50%, and could be enriched to >95% following cell sorting.

With the ability to obtain and grow primary cultures of mouse endothelial cells, we now have the capability to study interactions between these cells and cytotoxic effector cells. Studies are planned to evaluate the interactions of specific subpopulations of leukocytes, including NK cells, cytotoxic T cells and macrophages with these endothelial cells, and to examine the role of cytokines and/or other BRM in controlling them. These studies will be accomplished by first examining the binding of leukocytes to endothelial cells in vitro, following treatment with these specific agents. Future studies are also planned to investigate the penetration of endothelial cell monolayers and the underlying basement membrane in vitro. Using the modified Boyden chamber assay, both endothelial cell binding and diapedesis may be investigated by growing monolayer cultures of vascular endothelium on basement membrane-coated millipore filters. These filters will then be used in assays with effector cells on the upper side of the chambers, and various cytokines, BRM or chemotactic factors in the lower wells of the Boyden chamber. This technique will also permit us to evaluate physiological changes induced by effector cells on endothelium, including changes in shape and permeability, and to study the effects of BRM upon them. Knowledge gained from these studies could then be applied to in vivo mouse and human regimens to overcome the current limitations of cytokine and/or LAK therapies.

PUBLICATIONS

Futami H, Pilaro AM, Gruys ME, Back TT, Young HA, Wiltrout RH. in vivo distribution and cytokine gene expression by enriched mouse LAK effector cells, Biotherapy 1990;in press.

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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;43:372-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09348-01 LEI

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

Chemoprotective Effects of Recombinant Cytokines

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

PI:	R. H. Wiltrout	Head	LEI, NCI
Others:	G. Damia	Visiting Fellow	LEI, NCI
	M. MacPhee	Visiting Fellow	LEI, NCI
	H. Futami	Nakasone Fellow	LEI, NCI
	R. Jansen	Guest Researcher	LEI, NCI
	M. E. Gruys	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*

LMI, BRMP (F. Ruscetti, J. Oppenheim); OAD, BRMP (D. Longo); and Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC (K. Komschlies-McConville, J. Keller).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	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.)*

Selected cytokines have been evaluated for their ability to regulate the development of early hematopoietic progenitor cells and thereby protect mice from the acute toxicity of lethal doses of chemotherapy. Both rIL-1α and rTGFβ1 have been shown to have potent regulatory effects on leukocytes and bone marrow-stem cells. A single intra-arterial (ia) injection of TGFβ into normal BALB/c mice, transiently inhibits the proliferation of bone marrow cells by 30-50%. The formation of CFU-C from bone marrow is inhibited by about 30% and the formation of multipotential CFU-GEMM is inhibited by 50-100%. Because the number of CFU-GEMM per culture is quite low in normal marrow, we have also studied the ability of rTGFβ1 to inhibit the formation of colonies during the hyperproliferative phase of bone marrow repopulation that ensues about 7-9 days after the administration of 150 mg/kg 5FU. A single ia administration of rTGFβ1 also inhibits CFU-C by 30% and CFU-GEMM by about 50% in this setting. Subsequent studies have shown that the administration of single or multiple doses of 1-5 μg/mouse of rTGFβ1 consistently protects 10-30% of mice from the lethal toxicity of high dose (250 mg/kg) 5FU. The chemoprotective affects of rIL-1α are even more profound. The daily (5-7 days) administration of rIL-1α (≥ 10,000 U/day) protects 70-100% of mice from acutely toxic doses of 5FU (250 mg/kg) and cyclophosphamide (380-500 mg/kg). These increased doses of Cy were more effective at rendering mice tumor-free than were subacutely toxic doses of Cy. Surprisingly, a significant number of mice that were initially protected by IL-1α from Cy died about 60 days later as a result of pulmonary congestion and fibrosis. Current studies are focused on 1) the cellular and molecular mechanisms for the hematological and chemoprotective effects of rTGFβ1 and rIL-1α; 2) further investigation into improved therapeutic efficacy of intensified chemotherapy; and 3) the mechanism for the late Cy-induced pulmonary fibrosis and its prevention.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Giovanna Damia	Visiting Fellow	ETS, LEI, NCI
Martin MacPhee	Visiting Fellow	ETS, LEI, NCI
Hitoyasu Futami	Nakasone Fellow	ETS, LEI, NCI
Robert Jansen	Guest Researcher	ETS, LEI, NCI
M. Eilene Gruys	Biologist	ETS, LEI, NCI

OBJECTIVES

The overall objective of this project is to develop preclinical strategies by which doses of chemotherapy can be escalated in tumor-bearing rodents. The specific approach is to utilize cytokines with known potent hematological effects as chemoprotective agents. The specific objectives are as follows: (1) To determine the mechanism(s) by which rTGFβ_1 and rIL-1α modulate hematopoietic progenitor cell numbers and function in vivo; (2) To determine whether these cytokines are able to protect mice from acutely toxic doses of chemotherapy by virtue of their ability to spare critical progenitor cells; and (3) To determine whether the ability to deliver higher doses of chemotherapeutic drugs to tumor-bearing mice leads to increased therapeutic efficacy.

RESULTS

TGFβ_1 has been shown to inhibit bone marrow colony formation following in vitro treatment as well as after in vivo administration to normal mice. In collaboration with Drs. F. Ruscetti (LMI), J. Keller (PRI), and D. Longo (OAD), we investigated the differential effects of TGFβ_1 on various hematopoietic stem cells. Recombinant TGFβ_1 (rTGFβ_1) was administered locoregionally to mice via the femoral arteries before or after the administration of 5FU. When rTGFβ_1 was administered 24 hours before the assessment of CFU during the hyperproliferative state of the bone marrow that occurs 7-9 days after the administration of 150 mg/kg 5FU i.v., formation of multi-lineage (CFU-GEMM) colonies was inhibited by 51% and single lineage (CFU-C) colony formation was inhibited by 31%. Maximal effects were obtained with a dose of 5 μg/mouse. Pretreatment with TGFβ_1 24 hours before the same dose of 5FU inhibited the formation of CFU-C and CFU-GEMM at various time points after the 5FU with the degree of inhibition decreasing as a function of time after 5FU. Pretreatment, with rTGFβ_1 before administration of 5FU did not affect the formation of resting HPP-CFC, the earliest progenitor cell that is detectable in vitro. Further studies are now investigating the ability of rTGFβ_1 administered to mice 24 hours after the administration of 5FU, at a time when HPP-CFC progenitors should be stimulated to proliferate, to inhibit the formation of HPP-CFC. These results demonstrate that locoregional administration of rTGFβ_1 significantly inhibits the formation of both CFU-C and CFU-GEMM, with CFU-GEMM being the most sensitive. Further studies have shown that pretreatment of mice with single ia or multiple i.p. doses of rTGFβ_1 protects 10-30% of mice from an otherwise lethal dose of 5FU. IL-1 is a

multifunctional cytokine that plays also important roles in the regulation of immune responses and hematopoiesis. In collaboration with Dr. J. Oppenheim (LMI) recombinant human IL-1α (rhIL-1α) was used to protect normal and tumor-bearing mice from the acute toxicity caused by lethal doses of cyclophosphamide (Cy) and 5-fluorouracil (5FU). Pretreatment of mice for seven days with 10,000 U/day of rhIL-1α protected 70-100% of mice from the acute death induced by lethal doses of both Cy (380 mg/kg) and 5FU (250 mg/kg). In contrast, pretreatment of mice with either a single dose of rhIL-1α, or post-treatment with single or multiple doses of rhIL-1α demonstrated little or no chemoprotective effects. Pretreatment of mice with multiple doses of rhIL-1α increased the acute LD_{90} of Cy from 380 mg/kg to >500 mg/kg in normal mice, LD_{90} dose-modifying effect of at least 1.25. Acute chemoprotection by rhIL-1α was accompanied by a more rapid recovery from neutropenia and a less severe reduction in the number of bone marrow CFU-c. Mice bearing murine renal cancer (Renca) were also protected from the acute toxic effects of Cy (450 mg/kg) by pretreatment with rhIL-1α for seven days. Renca-bearing mice pretreated with rhIL-1α and either sublethal (300 mg/kg) or lethal (450 mg/kg) doses of Cy exhibited enhanced survival times over those of untreated Renca-bearing mice. Interestingly, the cause of death in Renca-bearing mice that died in spite of treatment with rhIL-1α plus 300 mg/kg Cy was recurrent tumor. On the other hand, most mice treated with rhIL-1α plus 450 mg/kg Cy had no detectable tumor. These results demonstrate that dose escalation of Cy permitted by pretreatment with rhIL-1α results in greater antitumor effects of Cy. In collaboration with Dr. K. Komschlies-McConville (BCDP, PRI), we are studying the role of the host immune system in the therapeutic synergy between sublethal Cy and rhIL-1.

Surprisingly, 10-50% of the mice that were successfully protected by pretreatment with rhIL-1α from the acute toxicity of Cy, died after day 50. These mice consistently presented with extensive pulmonary inflammation and fibrosis at death. An understanding of the mechanism responsible for this fibrosis, and an effective treatment for preventing or arresting it are desirable prior to the initiation of clinical trials of this approach. We hypothesize that the fibrosis is the result of a T-cell mediated autoimmune reaction initiated by the damage to lung tissue caused by cyclophosphamide in a manner similar to that reported for T-cells in bleomycin-induced pulmonary fibrosis. The chief difficulty with performing studies of this type is the prolonged time between the initiation of therapy and the death of the animals. To circumvent this delay, we have employed the measurement of collagen deposition in the lungs, as determined by increased hydroxyproline content. A chromogenic assay for total lung hydroxyproline content has been developed and its ability to measure pulmonary fibrosis confirmed by the detection of increased hydroxyproline levels in the lungs of bleomycin-treated mice. Using this assay, we have demonstrated a progressive increase in hydroxyproline content of the lungs from IL-1/Cytoxan treated mice. This increase was detectable by day 21 post-therapy, and its magnitude was dependent upon the dose of cytoxan. We plan to utilize this technique, supported by immunohistochemical studies of the lungs of affected animals, to determine the role of T-cells in this fibrosis through the use of both athymic mice and in vivo antibody depletion experiments. Following the identification of the cells involved in the development of fibrosis, neutralizing antibodies to various cytokines will be employed to determine the importance of the cytokines in the development of fibrosis. Appropriate drug and or cytokine-based therapies will then be devised and tested. The techniques

and capabilities developed for this project can also be used to study and hopefully prevent the development of fibrosis in other cytokine-based therapies such as peritoneal IL-2/IL-2+LAK cell treatment. Experiments to examine this form of fibrosis are also underway.

PUBLICATIONS

Goey H, Keller JR, Back T, Longo DL, Ruscetti FW, Wiltrout RH. Inhibition of early murine hematopoietic progenitor cell proliferation after in vivo loco-regional administration of transforming growth factor-β 1, J Immunol 1989;143:877-80.

Ruscetti F, Dubois C, Falk L, Jacobsen SE, Sing G, Longo DL, Wiltrout RH, Keller JR. In vivo and in vitro effects of TGFβ-1 on normal and leukemic hematopoiesis. In: Marsh J, ed. Proceedings from the Ciba-Geigy Symposium on TGFβ-1. Chichester: Wiley, 1990;in press.

SUMMARY REPORT

LABORATORY OF BIOCHEMICAL PHYSIOLOGY

October 1, 1989 through September 30, 1990

INTRODUCTION

The Laboratory of Biochemical Physiology (LBP) conducts research on the biochemical and molecular events related to the development of the malignant phenotype. An important aspect 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 our studies on the biochemical mechanisms of signal transduction pathways. Selected BRMs are studied in depth to determine the mechanisms by which they modulate cell growth/differentiation. In particular, the LBP has been applying molecular biology, biochemistry, and microinjection techniques to the studies of interferon/interleukin-6 (IL-6) and their receptors, ras and other guanyl nucleotide-binding proteins (G-proteins), phospholipase C (PLC), protein kinase C (PKC), and transcription and translation factors. The research effort can be divided into two principal avenues of investigation: 1. functional studies of ras proteins; and 2. the biochemical mechanisms of actions of interferons and IL-6.

With respect to AIDS-related research, we have been studying the negative regulation of HIV replication in monocytes. Also, we are screening potential anti-HIV agents and investigating their mode of action. The research activities are summarized below:

FUNCTIONAL STUDIES OF RAS PROTEINS

The ras gene family codes for a membrane-associated protein designated p21 which is closely related to the G-protein family of the signal transduction pathways. Cellular ras genes acquire transforming properties by single point mutations within their coding sequences, and the altered oncogenic ras genes are found in a significant fraction of human cancers and in experimentally induced animal tumors. Pharmacological and biological suppression of ras-mediated malignant transformations may be of some value in treating certain tumors involving oncogenic ras proteins. Therefore, the LBP has been investigating the biochemical and biological functions of ras proteins. Previously, we have been engaged in the characterization of the functional domains of ras proteins using linker insertion/deletion or site-directed mutagenesis. In the current studies, our research effort has been focused on the characterization of cellular targets involved in ras functions. Our major achievements are summarized as follows:

A. Characterization of a novel GDP/GTP exchange factor for ras proteins. Ras proteins have GDP/GTP binding and intrinsic GTPase activities. However, the oncogenic ras protein has approximately 1/10th of the intrinsic GTPase activity than its normal counterpart. It has been suggested that the reduced capability in GTP hydrolysis or GDP/GTP exchange may be directly responsible for ras transforming activity. This might result in an alteration of the ratio of active GTP-bound forms relative to inactive GDP-bound forms. A full understanding of ras function will require identification of mechanisms for interconverting these

two forms.

Although the physiological GTP concentration is at least 50- to 100-fold higher than that of GDP, the intrinsic GDP/GTP exchange rate of ras protein in vitro is rather slow. In order to determine how inactive GDP-bound p21 is reactivated, attempts were made to identify cellular protein(s) which stimulates the exchange reaction. Recently, we have purified from bovine brain a novel membrane factor, which markedly enhanced the guanine nucleotide exchange reaction of ras proteins. On SDS-polyacrylamide gel electrophoresis, the estimated molecular weight (Mr) of the factor is ~35 KDa. When the highly purified factor and excess unlabelled GTP were added to [^{3}H]GDP·p21 (normal or oncogenic p21), the nucleotide exchange rate was stimulated more than 25-fold. The net result would be to favor the active GTP·p21 form. We refer to this novel factor as ras guanine nucleotide exchange factor (rGEF). Our results suggest that the rGEF factor may control the rate limiting GDP/GTP exchange step in recycling of p21 in ras-mediated signal transduction. Modulation of rGEF activity would be expected to play an important role in ras-mediated transformation or could conceivably represent a novel class of oncogenic proteins. Biochemical characterization, molecular cloning, and functional studies of rGEF are in progress.

B. Functions of ras GTPase activating protein (GAP). While the intrinsic GTPase activity of normal ras protein is very weak, this reduced activity alone appears insufficient to maintain ras in the GDP-bound state under physiological conditions. This discrepancy has been partially reconciled by the identification of a cytoplasmic ras GAP, that stimulates the GTPase activities of normal ras proteins (>100-fold), but does not affect their activated or oncogenic counterparts. The net result would be to favor the GDP-bound form of the normal protein, while the oncogenic forms would persist in the active, GTP-bound form of the protein.

It has been shown that the effect of GAP upon ras GTPase activity is inhibited by mitogenically responsive lipids, which included phosphatidic acid, phosphatidylinositol phosphates, and arachidonic acid. The interaction of lipids with GAP/ras might be important in the regulation of cellular proliferation. In collaboration with Dr. Dennis Stacey, The Cleveland Clinic Foundation, we have shown that ras proteins with an insertion-deletion between amino acid residues, 97 and 108, are more sensitive to lipid inhibition. The results suggested that phospholipid metabolism might be related to the interaction between GAP and ras. Although GAP stimulates ras GTPase activity in vitro, its physiological functions in vivo have not been demonstrated. Thus, we have investigated the physiological roles of GAP in vivo, using Xenopus oocytes as a model system. Our results suggest that to induce full biological responses, ras proteins produce another signal(s), in addition to the one leading to diacylglycerol (DG) formation through GAP. Coinjection of GAP and anti-ras antibody (Y13-259) did not inhibit the GAP-induced increase of DG production suggesting that GAP action is downstream or independent of ras functions in the stimulation of DG formation. Our studies provide a new biological activity for GAP involving phospholipid metabolism and potentially a novel means of regulating phospholipid dependent protein kinases.

C. The role of small Mr G-proteins on ras functions and signal transduction. The α subunits of transducin, G_s, G_i, and G_o have Mr values between 39,000 and 52,000 and have GTP-binding and GTPase activities. This group of G-proteins is designated as large Mr G-proteins (lmg). There is another group of G-proteins with Mr values between 20,000 and 25,000 in mammalian tissues. These include the

three ras (H-, K-, and N-), three rho (-A, -B, and -C), ral, R-ras, four rab (-1,-2,-3, and -4), and three rap (-1A, -1B, and -2) genes. A similar G-protein gene encoding a protein with a Mr of 23,500, designated as SEC4, has been identified in yeast, but its occurrence in mammalian tissue is not known. Moreover, a G-protein with a Mr of 25,000, designated as Gp, and a G-protein with a Mr of 21,000, designated as ADP-ribosylation factor (ARF), have been purified from mammalian tissues. This group of G-proteins is designated as small Mr G-proteins (smg).

The physiological functions of lmg have been investigated extensively, and this family of G-proteins serves as transducers in transmembrane signalling. In contrast, the physiological functions of most smg have not been clarified. It is conceivable that smg are also involved in transmembrane signalling, but presumably in a manner different from that of lmg. Investigation of the functions of smg is essential in understanding various cell functions regulated by those smg proteins.

Although the physiological function of ARF remains to be elucidated, this G-protein has been shown to serve as a cofactor for the cholera toxin-dependent ADP-ribosylation of $G_{s\alpha}$. To investigate the function of ARF, we have isolated ARF cDNA clones from both human and Xenopus cDNA libraries. The DNA sequence is compared with that of bovine ARF. Comparison of the sequences of the coding regions between Xenopus and mammalian cDNAs shows good homology at the nucleotide and deduced amino acid level. Because of the conserved nature of ARF, we have used Xenopus oocytes as a model system to investigate the possible role of ARF in ras-mediated actions. Our results demonstrate the antagonistic effect of ARF on ras- and insulin-induced oocyte maturation. Biochemical mechanisms of ARF actions will be investigated.

D. The effect of PLC and PKC on ras functions. Previously, we have shown that the introduction of exogenous PLC-β and PLC-γ into NIH 3T3 cells induced DNA synthesis and morphologic transformation of growth-arrested fibroblast cells; and the ras protein is an upstream effector of PLC activity in PI signal transduction. Ras p21 proteins are important elements in the signal transduction pathways involved in cell growth. One pathway may be coupled with calcium and phospholipid-dependent PKC. It has been shown that transfection of NIH 3T3 fibroblasts with PKC cDNA enhances tumorigenicity and produces changes in cellular morphology and other growth properties. The involvement of functional PKC in the mitogenic response to the H-ras oncogene product has also been reported. In addition, ras oncogene-transformed cells showed the accumulation of DG and profound attenuation of a 80 KDa endogenous substrate of PKC. Biochemical mechanisms of actions of PKC in ras-mediated functions remain to be elucidated. Xenopus oocytes have become a convenient system for biochemical analysis of PKC/ras functions. As described above, the level of DG, an endogenous activator of PKC, is rapidly increased following the introduction of the oncogenic ras protein into Xenopus oocytes. Therefore, it is of interest to investigate whether PKC affects oncogenic ras protein-induced biological responses in oocytes. Our results indicate that microinjected PKC potentiates ras protein action by increasing the rate of induction of both oocyte maturation and ribosomal S6 protein phosphorylation. The synergistic effect of PKC on ras-induced S6 phosphorylation is due to an overall increase in phosphorylation of S6 derivatives rather than a qualitative change in species of phosphorylated S6 derivatives. The molecular mechanism underlying the synergistic effect of PKC on the ras action is not clear at present. In any case, our data further emphasize

the regulatory role of ras proteins and PKC in signal transduction in early development. As for the role of PKC in Xenopus laevis development, it has been shown that PKC mediates neuronal induction and enhances insulin-induced oocyte maturation.

E. Functional relationships between ras and other oncogenes. Transfection, microinjection, electroporation, and scrape-loading techniques have been used for the studies of oncogenes and their gene products. Among these techniques, microinjection assay requires only small quantities of samples. Therefore, it has become an established method in our laboratory for the functional studies of a variety of oncogenes. We have previously shown that microinjection of the anti-ras monoclonal antibody, Y13-259, prevents NIH 3T3 cells from entering the S-phase. These results imply that ras gene is required for a normal essential function of NIH 3T3 cells. Identification of the neutralizing antibody, Y13-259, provided an opportunity to study the interaction between ras genes and other oncogenes. It has been shown by microinjection of the Y13-259 antibody that transformation of NIH 3T3 cells by fes, fms, and src oncogenes depends on ras functions, while transformation by mos and raf oncogenes appears to be independent of ras functions. It is possible that raf/mos acts downstream of ras or alternatively raf/mos transforms cells via a pathway different from ras. In collaboration with Dr. Ulf Rapp (NCI), attempts have been made to unveil the functional roles of raf gene products. These studies require the availability of raf proteins, and we have expressed the proteins in E. coli. The carboxyl-terminal portion of the human c-raf-1 gene was fused to the amino-terminal portion of the bacteriophage cII gene on the expression vector pJL6. The truncated raf protein was partially purified and microinjected into NIH 3T3 cells. Our studies showed that raf protein itself is sufficient to induce a transformed morphology. In addition, the injected protein stimulated quiescent cells to enter the S phase of the cell cycle. This result clearly demonstrates that the raf gene functions directly through its protein product. In collaboration with Dr. Steve Giardina (PRI), we are in the process of identifying neutralizing anti-raf antibodies in order to establish the signal transduction pathway(s) involving ras/raf actions. Besides anti-raf antibodies, anti-fos antibodies were prepared at the BRMP. Among the available antibodies we have identified several neutralizing anti-fos antibodies and demonstrated that fos is required for serum-stimulated growth of NIH 3T3 cells. The availability of ras/raf oncogene proteins and neutralizing anti-ras/fos antibodies has prompted us to consider the studies of functional relationships among various oncogenes in NIH 3T3 cells. Oncogenes can be categorized into four classes based upon biochemical functions or subcellular localization of the gene product. One class of oncogene products are related to growth factors and their receptors. A second class of oncogene products are related to G-proteins involved in signal transduction. Cytoplasmic serine/threonine kinases make up a third class of oncogenes. The final grouping of oncogene products are nuclear proteins involved in transcriptional regulation. The fos and jun oncogene products have been found to be associated as components of the AP-1 complex, which regulates transcription of specific mRNAs. In addition to transcriptional factors, we have recently investigated the role of eukaryotic translation initiation factors (eIFs) in the control of cellular proliferation and demonstrated that microinjection of eIF-4E or eIF-4F induces DNA synthesis and morphologic transformation in NIH 3T3 cells. One of the possible mechanisms by which excess eIF-E/eIF-F in the cells evokes malignant transformation is that translation of certain oncogenes is regulated by the availability of eIF-E/eIF-F. The functional relationships between eIFs and oncogenes will be further investigated.

THE BIOCHEMICAL MECHANISMS OF ACTIONS OF INTERFERONS AND IL-6

The interferons (IFNs) are a family of proteins with potent antiviral, antiproliferative, and immunomodulatory activities. In order to elucidate the multiple facets in the mechanisms of IFN action, the LBP has been concentrating on studies of the structure, function, and modulation of IFN-α and IL-6 receptors, the intra-cellular role of IFN-γ, signal transduction pathways in the action of IFN, modulation of the expression of specific proteins by IFNs, and the biology of IL-6 system. Most studies have been summarized in the previous annual reports. In the past year, we have been focusing on the studies of the IL-6 system.

We have shown that IFN-α down-regulates the IL-6 receptor on myeloma cells and are currently examining whether the growth inhibition exerted by IFN-α on myeloma cells is due to down-regulation of the IL-6 receptor. These studies may have clinical relevance for the treatment of multiple myeloma. We have also investigated the expression of the IL-6 receptor on monocytic and myeloma cell lines by affinity cross-linking and Scatchard analysis. Our results indicate that myeloma cells express a unique high affinity IL-6 receptor which has not been described before. Characterization of this novel high affinity IL-6 receptor is in progress.

AIDS-RELATED RESEARCH

We have been working on the regulation of HIV gene expression and the identification of potential anti-HIV agents. Our previous results showed the existence of negative regulatory cellular factor(s) in the restricted state of HIV-infected monocytoid leukemic cell line, THP-1. In vitro transcription and DNA-binding gel retardation assays have been used to study the molecular mechanism of HIV gene regulation in monocytes/macrophages. These studies demonstrate the involvement of the 65 + 50 KDa NF-κB heterotetramer in the negative regulation of chronic low-level HIV expression in monocytes. Recent reports suggested a role for the negative regulatory element (NRE) of HIV-LTR in the regulation of HIV gene expression. Therefore, it is important to identify the cellular factors interacting with NRE. Using the Southwestern screening technique, we have isolated several cDNA clones encoding sequence-specific (IL-2/IL-2R promoters and NRE of HIV-LTR) DNA binding proteins. The deduced amino acid sequences reveal a typical zinc-finger and leucine zipper structure characteristic of DNA-binding proteins. The sequence data indicated that these clones represent newly discovered genes. The functions of those proteins in the transcriptional regulation of HIV are currently under investigation.

Finally, we tried to isolate new inhibitors of HIV infection and replication from traditional Chinese herbs. In collaboration with Dr. Sylvia Lee-Huang, New York University Medical School, a new inhibitor of HIV has been isolated and purified to homogeneity from the seeds and fruits of the Chinese medicinal plant Momordica charantia. This compound, MAP 21, is a basic protein of 30 KDa. It exhibits a dose-dependent inhibition of HIV-1 infection and replication, as measured by quantitative focal syncytium formation of a cell monolayer, viral core protein p24 expression, and viral-associated reverse transcriptase activity. No cytotoxicity or cytostatic activity was found under the assay conditions. These data suggest that MAP 21 is a potentially therapeutic agent in the treatment of HIV-1 infection. The N-terminal 44 amino acid sequence of MAP 21 has been determined. Comparison of this sequence to those in a database revealed homology

MAP 21, we have identified two other Chinese herbs with inhibitory effects on HIV infectivity and replication, i.e. Baicalin and Phyllanthus. The active component of Baicalin has been purified from the Chinese herb Scatellaria baicalensis georgi, and its chemical structure has been determined as a flavone derivative. Phyllanthus is an aqueous extract from the Chinese medicinal plant, Phyllanthus urinaria L.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09299-04 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Inhibition of Infectivity and Replication 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-FCRDC (B. Li); LBS, LTCB, NCI-FCRDC (P. Nara)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	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.)*

Previously, we have shown the inhibitory effect of chloroquine on the infectivity of human immunodeficiency virus (HIV-1) in human T-cell lines. The data suggests that chloroquine inhibition of infectious virus production is most likely due to interference with terminal sialylation in the trans-Golgi network. We will test this drug alone or in combination with other drugs on primary cell cultures. Experiments are in progress to establish a primary peripheral blood lymphocyte/monocyte culture system for this purpose and to develop quantitative bioassays for detection of primary cultures that are infected with HIVs.

In addition, attempts have been made to identify potential anti-HIV agents from traditional Chinese herbs. A new inhibitor of HIV infection and replication has been isolated and purified to homogeneity from the seeds and fruits of the Chinese medicinal plant Momaordica Charantia. This protein, MAP 21, has been sequenced and sequence comparison revealed homology with the ricin A chain and with several forms of trichosanthins. No cytotoxicity was found under the culture conditions for anti-HIV assay. Therefore, MAP 21 could be a potential anti-AIDS drug. Besides MAP 21, we have identified two other Chinese herbs with anti-HIV activity, i.e. Baicalin and Phyllanthus.

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. The work has been extended to include the identification of potential anti-HIV agents inhibiting other steps of the HIV life cycle.

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. We have identified the following three Chinese herbs with anti-HIV activity: 1. MAP 21 is a ribosome-inactivating protein purified from the Chinese medicinal plant, Momordica Charantia, which has been purified to homogeneity and sequenced, and molecular cloning of MAP 21 is in progress; 2. Baicalin is a flavone derivative; and 3. Phyllanthus is an aqueous extract from the Chinese herb, Phyllanthus Urinaria L.

OBJECTIVES AND FUTURE PLANS

The intracellular glycosylation and proteolytic cleavage of HIV 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 and in primary cell cultures. 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. To facilitate these studies, we will develop quantitative bioassays for detection of primary cultures that are infected with HIVs. The mode of action of MAP 21, Baicalin, and Phyllanthus on the inhibition of HIV infection and replication will be investigated.

PUBLICATIONS

Tsai WP, Nara PL, Kung HF, Oroszlan S. Inhibition of human immunodeficiency virus infectivity by chloroquine, AIDS Res Hum Retroviruses 1990;6:481-9.

Lee-Huang S, Huang PL, Nara P, Chen HC, Kung HF, Huang PH, Huang HI, Huang, PL. Map 30: A new inhibitor of HIV infection and replication, FEBS Lett:1990, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09300-04 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Intracellular Activities of Cytokines

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	Section Head	CMIS, LMI, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI, NCI-FCRDC (M. Smith, Y. Liu, J. Keller)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	0.0	3.0

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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.)*

Cytokines like IFN-γ and TNF-α have pleotrophic effects on target cells. The mechanism(s) by which these cytokines exert their biochemical modulating effects on cells has not been defined conclusively. Investigators have described the activation of second messenger systems following ligand-receptor interaction, and it has been demonstrated that ligand-receptor complexes are internalized by receptor-mediated endocytosis into endosomes and lysosomes within the cell. We considered the possibility that these cytokines, after internalization, could act intracellularly to modulate the biochemical process of the cell. This hypothesis was tested by directly introducing cytokines into the cytoplasm of the cell via microinjection. We determined that IFN-γ and TNF-α do have intracellular roles in that injection of human IFN-γ into murine macrophages induced Ia antigen expression on the cell surface and that injection of TNF-α induced rapid cytotoxicity, killing target cells through the activation of a cytoplasmic endonuclease. IL-1 and IL-2 were found not to have an intracellular biological role, since injection of IL-1/IL-2 did not result in the induction of DNA synthesis normally seen for the binding of these cytokines to their receptors.

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

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Scott Durum	Senior Staff Fellow	LMI, NCI
Howard Young	Section Head	CMIS, LEI, 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).

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.

METHODS

The following techniques were used: 1. microinjection; 2. tissue culture; 3. protein purification; 4. immunological staining; 5. in situ hybridization; and 6. enzyme assays.

MAJOR FINDINGS

Human IFN-γ induced Ia expression on the surface of murine macrophages after microinjection, even though the addition of human IFN-γ into the culture median of murine macrophages has no effect on Ia expression due to the failure of human IFN-γ to bind to the murine IFN-γ receptors. This observation suggests that IFN-γ has an intracellular biochemical role and that human and murine IFN-γ share common domain(s) that mediates this intracellular activity. The injected IFN-γ was shown to activate the release of granulocyte/macrophage-colony stimulating factor (GM-CSF) from injected cells that in turn induced Ia antigen expression on neighboring cells. The Ia induced by IFN-γ injection could be blocked by neutralizing murine anti-GM-CSF antibody in the culture media. Conditioned media from IFN-γ treated and injected cultures were shown to contain GM-CSF activity. Our findings provide direct evidence that IFN-γ internalization may be critical to its mode of action, and extracellular peptide ligands can enter a cell and elicit a biologic response.

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 fibroblast cells (L6) 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. L6 fibroblasts have an intracellular mechanism that protects the cell from the cytotoxicity of TNF. Also, a TNF-resistant L929 cell line was not affected 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.

Cytokines and polypeptide hormones with pleiotrophic functions may exert their effects not only through ligand-receptor binding, but also through interaction with intracellular targets distinct from surface receptors. We conclude that some cytokines can have an intracellular role, suggesting that receptors may have in addition to transducer roles, a role in transporting hormones to an intracellular site where they have a biochemical function.

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 purify and characterize the TNF activated endonuclease in order to clone it.

4. To investigate the mechanism of Ia expression induced by IFN-γ through the release of preformed GM-CSF.

PUBLICATIONS

Smith MR, Munger WE, Kung HF, Takacs L, Durum S. Direct evidence for an intracellular role for tumor necrosis factor-α: Microinjection of tumor necrosis factor kills target cells, J Immunol 1990;144:162-9.

Smith MR, Muegge K, Keller JR, Kung HF, Young HA, Durum S. Direct evidence for an intracellular role for IFN-γ: Microinjection of human IFN-γ induces Ia expression on murine macrophages, J Immunol 1990;144:1777-82.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09301-04 LBP

PERIOD COVERED: October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Phospholipase C and Cytoplasmic Kinase in Signal Transduction

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

PI: H. Kung Chief LBP, NCI

COOPERATING UNITS *(if any)*: BCDP, PRI, NCI-FCRDC (T. Kamata, M. Smith, M. West); IR, NHLBI, NIH (S. Rhee); LVC, NCI-FCRDC (U. Rapp)

LAB/BRANCH: Laboratory of Biochemical Physiology

SECTION:

INSTITUTE AND LOCATION: NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	0.0	3.0

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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.)*

Ras proteins have been identified to be associated with the development of a great diversity of human tumors. Biochemically ras proteins are believed to play important roles in signal transduction. Several reports have suggested that ras-like G-proteins modulate the activity of phosphoinositide-specific phospholipase C (PLC)-dependent signalling pathways. Our data indicate that PLC activity is necessary for ras-mediated induction of DNA synthesis in NIH 3T3 cells and that ras may function as a G-protein-like molecule in inositol phospholipid signal transduction.

Raf protein possesses serine/threonine kinase activity and is localized in the cytoplasm. In order to investigate the functional relationship between raf and ras/PLC in signalling pathways, we have expressed raf proteins in E. coli. Microinjection studies show that truncated raf protein itself is sufficient to induce transforming activities, although the full-length raf protein is not active by itself. This result clearly demonstrates that the raf gene functions directly through its protein product. The regulation of raf protein transforming activities is currently under investigation.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI

OBJECTIVES

The objectives of this project are to study the biological roles 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

Phosphoinositide-specific phospholipase C (PLC) was shown to be mitogenic after injection into NIH 3T3 cells, suggesting that PLC is an intermediate in the biochemical pathways used by the cell to proliferate. This observation had been made previously for the ras protein. In addition, a neutralizing monoclonal antibody made against ras was microinjected into primary and established cell lines and was shown to block serum-induced DNA synthesis, demonstrating an absolute requirement for ras proteins in order for a cell to proliferate. We identified a mixture of monoclonal antibodies made against phosphoinositide-specific PLC that also blocked serum-induced DNA synthesis in NIH 3T3 cells. When ras and PLC proteins were co-injected with their neutralizing monoclonal antibodies, in every possible combination, the mitogenic signal from ras and PLC were blocked by anti-PLC monoclonal antibodies. When ras and PLC were co-injected with neutralizing anti-ras monoclonal antibody only the transforming signal from ras protein was blocked, while the signal from PLC was not. These results suggest that the ras protein is an upstream effector of PLC activity in phosphoinositide-specific signal transduction and that PLC activity is required for ras-mediated induction of DNA synthesis.

Cytoplasmic factors involved in signal transduction include several serine/threoine kinases that are believed to transmit the proliferation signal into the nucleus of the cell. One such cytoplasmic kinase, raf, was originally identified as the transforming sequences of the murine sarcoma virus 3611. Full length and 5' deletion mutants of the c-raf gene were cloned and expressed in E. coli. Induced protein was purified and microinjected into quiescent NIH 3T3 cells. Full length raf protein was found not to be transforming or to induce DNA synthesis in these cells, while deletion mutants were effective at inducing both transformation and DNA synthesis. The full length raf has been recently shown to become transforming and will induce DNA synthesis if co-injected with C kinase. This result supports the hypothesis that the normal raf protein assumes a conformation with a hinge region where the kinase domain is covered by amino terminal amino acids and the phosphorylation of the amino termini results in opening the molecule, exposing the kinase domain for activity.

FUTURE PLANS

To use the microinjection assay for continued screening of antibodies against various BRMs that modulate growth and differentiation of cells.

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.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09302-04 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on Novel Ras Guanine Nucleotide Exchange Factor in a 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: M. Maeno	Visiting Fellow	LBP, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI, NCI-FCRDC (T. Kamata, Y. Huang, M. West, G. Kovacs)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

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 unreduced type. Do not exceed the space provided.)*

Mammalian ras p21 proteins like other G-proteins bind GDP and GTP. It is assumed that a growth signal stimulates the conversion of p21·GDP to p21·GTP at the plasma membrane and that active p21·GTP interacts with an effector molecule to transmit an internal signal. However, it has not been clarified how inactive GDP-bound p21 is reactivated. Studies were carried out to search for the factor(s) regulating this particular step in p21 recycling. We have identified a novel membrane factor, which markedly stimulated the guanine nucleotide exchange activity of ras proteins. The ras guanine nucleotide exchange factor (rGEF) was purified from bovine brain to near homogeneity by 4-step column chromatographies. The purified rGEF exhibited a single major 35kd protein. rGEF increased the exchange rate of GDP in normal and oncogenic ras proteins to 30 - 40 fold. Since the factor was free from GDP/GTP binding activity and non-specific GDP hydrolysis activity, we hypothesize that rGEF may regulate GDP/GTP exchange reaction of ras proteins in response to external signals. To further characterize this factor, we are currently trying to clone the gene encoding rGEF. These findings are extremely interesting, implying that the biological activity of ras proteins may be regulated by a guanine nucleotide exchange system in a manner similar to translation elongation factor EF-Tu or the G-proteins of adenylate cyclase system.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Mitsugu Maeno	Visiting Fellow	LBP, NCI

INTRODUCTION

The ras gene family codes for a membrane associated protein designated p21 which is closely related to the G-protein family of the signal transduction pathways. Cellular ras genes acquire transforming properties by single point mutations within their coding sequences, and the altered oncogenic ras genes are found in a significant fraction of human cancers and in experimentally induced animal tumors. Pharmacological and biological suppression of ras-mediated malignant transformations may be of some value in treating certain tumors involving oncogenic ras proteins. Therefore, the LBP has been investigating the biochemical and biological functions of ras proteins. Previously, we have been engaged in the characterization of the functional domains of ras proteins using linker insertion/deletion or site-directed mutagenesis. In the past year, our research effort has been focused on the characterization of cellular targets involved in ras functions, especially the GDP/GTP exchange factor for ras proteins.

OBJECTIVES

The objectives of this project are to study the regulatory mechanism for ras activity in growth-factor-mediated signal transduction and to explore the mechanism of ras oncogene induced cell transformation.

METHODOLOGY

1. Protein purification

2. Biochemical analysis

MAJOR FINDINGS

Recently ras GTPase activating protein (GAP) has been proposed to play a role as a signal terminator for normal ras p21 proteins. However, in this model it is not clear how inactive GDP·p21 is converted to active GTP·p21 in response to external stimuli, such as growth signals.

We have tested whether bovine brain tissue extracts contain the factor capable of stimulating the guanine nucleotide exchange reaction of p21 proteins. Detergent-solubilized membrane extracts greatly accelerated the reaction, whereas cytoplasmic extract had no significant effect. This suggests that the factor is associated with membranes. By utilizing bovine brain membrane fractions as starting materials, we have purified the factor to near homogeneity by four steps of column chromatographies.

The purified factor exhibited a single major 35 kd protein and was free from GDP/GTP binding activity. The factor stimulated the rate of nucleotide exchange reaction in either normal or oncogenic ras proteins to 30 - 40 fold. This affect was completely blocked by the ras neutralizing antibody, Y13-259. The nucleotide exchange activity of other G-proteins, such as transducing and translation elongation factor EF-Tu, was not affected by the factor. Furthermore, the estimated molecular weight of the factor is different from any known nucleotide exchange factor. Therefore, we designated the factor as ras-guanine nucleotide exchange factor (rGEF). We propose that the rGEF may control the rate limiting GDP/GTP exchange reaction of ras proteins. Peptides of purified rGEF were microsequenced. Computer analysis showed that there is no significant identity in amino acid sequence between microsequenced peptides of rGEF and known proteins. In order to isolate the gene encoding rGEF, we are in the process to construct and to screen bovine brain cDNA library with oligonucleotide probes.

FUTURE PLANS

We will continue to isolate the gene encoding rGEF from bovine brain cDNA library by using oligonucleotide probes and anti-peptide antibodies against rGEF.

To establish the physiological activity of rGEF, we will perform biological and biochemical studies of rGEF by utilizing isolated genes and overexpressed rGEF recombinant protein. These include: 1. the determination of functional domains of ras/rGEF molecules involved in ras-rGEF interaction; and 2. biological studies using anti-rGEF neutralizing antibodies or anti-sense rGEF DNA oligonucleotides.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09312-04 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Action and the Receptors for Interferons and IL-6

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

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	.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.)*

Previously, we have investigated the mechanism of action of interferons (IFNs). In our present research we are focusing on the biochemical studies of interleukin-6 (IL-6). We have identified two IL-6 binding proteins with apparent Mr of 80 and 108 kDa. These two proteins which appear to have different affinities for IL-6 can be visualized as an 100 and a 130 kDa receptor complex upon affinity-crosslinking with iodinated recombinant IL-6. Our studies indicate that the IL-6 binding protein contained within the 130 kDa complex comprises a novel, high-affinity IL-6 receptor which has not been described previously. We observed that the expression of both IL-6 receptor molecules varies with cell lineage. In addition, we have demonstrated that the 108 kDa receptor mediates the internalization of its ligand. These findings are important for the elucidation of the signalling pathway of IL-6, which is currently poorly understood. We also investigated whether IFN could modulate the expression of the IL-6 receptors, and our data indicate that IFN down-regulates the IL-6 receptors. Since IFN causes growth arrest of human myeloma cell lines which express both IL-6 receptors, we will test the notion whether the antiproliferative mode of action of IFN in myeloma cell lines is due to the disruption of a functional IL-6 receptor-signalling pathway. Our main research effort, however, will focus on the biochemical and molecular elucidation of the structure and function of the 108 kDa IL-6 receptor. Purification and molecular cloning of the receptor are in progress.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Gerald Princler	Chemist	LBP, NCI
Michael Schwabe	Visiting Fellow	LBP, NCI

OBJECTIVES

IL-6 has recently been shown to play a central role in the mediation of several host defense mechanisms. The actions of IL-6 include the terminal differentiation of activated B-cells into immunoglobulin secreting plasma cells, a co-stimulatory role in T-cell activation, and the induction of a hepatic acute phase response. Most interestingly, IL-6 might function as an autocrine growth factor in multiple myeloma with an opposite effect (antiproliferative) in monocytic leukemia cells. We, therefore, undertook a detailed biochemical analysis of the IL-6 receptor on monocytic and myeloma cell lines to investigate whether this differential IL-6 effect may be explained by the expression of distinct molecular forms of the IL-6 receptor.

MAJOR FINDINGS

The interaction between IL-6 and its receptor was originally described as the binding to a single class of high affinity receptors. This initial observation led to the identification of a single chain IL-6 binding protein, which has been designated p80. Shortly thereafter, the p80 receptor was cloned. The amino acid sequence of the p80 IL-6 receptor, however, did not reveal any clues for the potential signal transduction mechanism, such as potential phosphorylation sites or intrinsic kinase activities. Subsequently, another molecule (gp 130) was identified which is believed to contain a single transducing moiety for IL-6. gp 130, however, was found not to bind IL-6 by itself and is, therefore, considered to be an IL-6 receptor-associated molecule which may initiate an IL-6 response by the interaction with an IL-6 - p80 receptor complex.

Since IL-6 has been shown to act as a growth factor in one in vitro system (plasmacytoma) but as a growth inhibitor in others (monocytic leukemia and breast cancer cell lines), we reasoned that these apparent differences could be accounted for by distinct IL-6 receptors on the respective cell lines. To test this notion we iodinated human recombinant IL-6 to perform a biochemical analysis of the IL-6 receptor on monocytic and myeloma cell lines. A Scatchard analysis indicated that IL-6 interacted on monocytes with a single class of receptor molecules with a Kd of 200 pM, whereas myeloma cells appeared to express two distinct affinity sites giving to a high-affinity (Kd 10 pM), as well as a low affinity component (Kd 240 pM). In order to further investigate the molecular nature of the IL-6 receptors on myeloma cells, we performed affinity cross-linking experiments to visualize the IL-6 receptors. Our data indicate that monocytic cell lines express a single IL-6 binding protein, which can be detected as a 100 kDa complex by SDS-PAGE. On myeloma cell lines, however, we detected two distinct IL-6 receptor complexes with apparent Mr of 100 and 130 kDa. By subtracting the molecular weight of the iodinated IL-6 (22 kDa), these results

translate into a 88 kDa receptor on monocytes and a 78, as well as a 108 kDa receptor on myeloma cells. Since we could rule out that the appearance of the 130 kDa band is due to the crosslinking of a dimeric IL-6 molecule to the p80 receptor, our results suggested that myeloma cells express a second IL-6 binding protein. In order to investigate whether the high-affinity sites detected by Scatchard analysis may be attributable to either the 100 or 130 kDa complex, we undertook affinity-crosslinking experiments with increasing concentrations of iodinated IL-6. We observed that at low IL-6 concentrations, relatively more label was incorporated into the 130 kDa complex whereas at higher IL-6 concentrations, more label was detected within the 100 kDa complex. These data indicated that the IL-6 binding protein contained within the 130 kDa complex most likely represents a high-affinity IL-6 receptor. Interestingly, a Northern blot analysis performed with a probe specific for the 80 kDa receptor revealed the expression of a single 5.5 kb message in all cell lines tested so far which bind IL-6. At present, we do not know whether the second IL-6 receptor expressed on myeloma cells derived by either differential splicing of the p80 mRNA or by post-translation modifications of the receptor protein or the p108 receptor may be derived from different gene.

Furthermore, we have shown that the treatment of myeloma cells with IL-6 leads to internalization of IL-6, an event which is preceded by the down-regulation of the 108 kDa receptor. A Scatchard analysis performed with IL-6 treated cells indicated a preferential loss of the high-affinity sites. This finding was further substantiated by affinity-cross-linking experiments, in which the 130 kDa complex becomes barely detectable on cells pretreated with IL-6. Besides IL-6, IFN at doses of 1000 U/ml also led to the down-regulation of the IL-6 receptor, as detected by affinity cross-linking and Northern blot analysis.

FUTURE PLANS

Our future plans will focus on the elucidation of the molecular structure of the 108 kDa IL-6 receptor. We hope to achieve this goal by purifying the receptor molecule, by the subsequent generation of anti-receptor antibodies and the molecular cloning of a cDNA encoding for the receptor molecule.

PUBLICATIONS

Boublik M, Moschera JA, Wei C, Kung HF. Conformation and activity of recombinant human fibroblast interferon, J Interferon Res 1990;10:213-9.

Bushmeyer SM, Schiller JH, Ruzicka FJ, Princler GL, Faltynek CR, Borden EC. Modulation of interferon receptor expression during combination interferon-β_{ser} and interferon-γ treatment, Cancer Res 1990;50:26-31.

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DeBenedetti F, Falk LA, Ruscetti FW, Colburn NH, Faltynek CR, Oppenheim JJ. Inhibition of PMA-induced transformation of JB6 cells by transforming growth factor-β, Cancer Res 1990; 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, Blood 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09315-03 LBP

PERIOD COVERED: October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: HIV Latency and Roles of Cellular Factors in 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:	I. Calvert	Chemist	LBP, NCI
	F.W. Ruscetti	Microbiologist	LMI, NCI
	C. Samalekos	Visiting Fellow	LBP, NCI
	J. Mikovits	Microbiologist	LMI, NCI

COOPERATING UNITS *(if any)*: BCDP, PRI, NCI-FCRDC (Raziuddin, M. West)

LAB/BRANCH: Laboratory of Biochemical Physiology

SECTION:

INSTITUTE AND LOCATION: NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	2.0

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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.)*

There is substantial evidence suggesting that the macrophage/monocyte serves as a reservoir for HIV infection and plays an important role in the pathogenesis of AIDS. HIV-1 replication is regulated by virally encoded proteins, as well as by cellular transcriptional factors. To understand the mechanisms of viral latency and restricted low level expression we have developed HIV infected monocytic cell lines, THP-1 as a model system where expression is observed at various levels; a. complete latency, b. restricted low level expression, and c. productive infection. 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 THP-1. We are currently studying the DNA element(s) involved in the negative regulation of HIV expression. Using Southwestern screening technique, we have cloned cDNAs encoding for the proteins that bind to the negative regulatory element (NRE) of the HIV-LTR. 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. With regard to the roles of NF-κB in HIV gene expression, we have used purified NF-κB and demonstrated the involvement of the 65 + 50 kDa NF-κB heterotetramer in the negative regulation of HIV expression in monocytes. The protein, IκB, has been shown to be an inhibitor of NF-κB binding activity. IκB binds specifically to the 65 kDa subunit of NF-κB, blocks heterotetramer formation and rapidly dissociates the complex of NF-κB with its cognate DNA. Studies are in progress to prove that IκB is an inhibitor involved in this negative regulation of HIV transcription.

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

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Francis W. Ruscetti	Microbiologist	LMI, NCI
Charalambos Samalekos	Visiting Fellow	LBP, NCI
Judy Mikovits	Microbiologist	LMI, NCI

INTRODUCTION

The 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

The following techniques were used: 1. in vitro nuclear run-off assay; 2. in vitro transcription assay; 3. DNA-binding gel retardation assay; and 4. molecular cloning of sequence-specific DNA binding proteins (a southwestern technique using a p^{32} labelled site-specific DNA probe).

MAJOR FINDINGS

Using an entire enhancer plus TAR region in a gel binding assay our preliminary results showed a dramatic difference in the binding pattern of the two states of HIV infection in monocytes. Furthermore, we have demonstrated the existence of negative regulatory factor(s) in the restricted state of HIV infected monocytes, THP-1. To investigate the specific DNA sequences involved in DNA-protein complex formation, gel binding assays were performed with smaller DNA segments within the enhancer and TAR regions. The DNA probe that bound to the nuclear extracts generating the specific DNA-protein complexes was derived from a 11-base pair direct repeat motif in the enhancer region of HIV-1 LTR. These sequences are also present in the κ-immunoglobulin gene enhancer region (Nature: 326:326,1987).

To study whether the specific complexes formed were due to the direct involvements of NF-κB, gel retardation assays of nuclear extracts were performed in the presence of purified NF-κB (a generous gift from Dr. S. Ghosh, Whitehead Institute, Cambridge, MA). Two gel retarded complexes were observed with the nuclear extract of productively infected cells, as well as with purified NF-κB. The upper complex is due to 50 + 65 kDa heteromer, whereas the lower complex is the 50 kDa homomer, both of which are due to NF-κB. This upper 50 + 65 kDa heteromer is totally missing from the restrictedly infected monocytes. However, when mixed with increasing amounts of purified NF-κB, the nuclear extracts from restricted state regenerate the upper 50 + 65 kDa heteromer complex. A NF-κB inhibitor, named IκB, is normally present in the cytosol. NF-κB could only be inactivated by IκB when p65 was bound. The inhibition of 50 + 65 kDa heteromer complex formation by IκB is reversible by deoxycholate (DOC) treatment. Treatment of restricted state nuclear extracts with DOC showed a dramatic increase in 50 + 65kDa heteromer formation. This result makes it most likely that in our restricted state, a IκB-like inhibitor of NF-κB is present, which in turn prevents the active binding to the enhancer-promoter element leading to a down-regulation of HIV transcription.

Molecular cloning and characterization of a novel sequence-specific DNA-binding protein recognizing the negative regulatory element (NRE) region of HIV-1 LTR. Transcriptional regulation depends on the sequence specific interaction of transactivating factors with cis-activating DNA elements. A majority of these proteins binds selectively to distinct transcriptional control elements and are, thereby, implicated in regulating the activity of their target genes. Deletion of the entire negative regulatory sequences resulted in many-fold increase in LTR-directed gene expression (Cell 41:813,1985). In our studies with restricted HIV expression, the viral expression is negatively regulated in such a manner as to escape immune surveillance and still be capable of transmitting the virus to T-cells. Based on these studies we anticipate that the NRE sequences, besides other elements of HIV-LTR, are responsive for the negative regulatory effect on gene expression directed by HIV-LTR.

We have isolated a cDNA clone which encodes a sequence-specific DNA-binding protein specifically recognizing a 60 bp motif within the NRE region. This is the first sequence specific DNA-binding protein reported to recognize the NRE region. The cDNA represents a single copy gene that is expressed strongly in a variety of cell types tested implying that the gene is expressed in a ubiquitous fashion. The recombinant protein is highly rich in gln/pro and ser/thr, which are characteristics of a majority of sequence specific DNA-binding proteins and transcriptional factors. Studies on the functional roles of this protein in gene regulation are in progress.

FUTURE PLANS

Our future plans include: 1. further characterization 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; and 5. studies of HIV gene regulation in fresh monocytes.

PUBLICATIONS

Mikovitz J, Raziuddin, Gonda M, Ruta M, Lohrey NC, Kung HF, Ruscetti FW. Negative regulation of HIV replication in monocytes, J Exp Med 1990;171:1705-20.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09316-03 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

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: J. Zhao	IRTA Fellow	LBP, NCI

COOPERATING UNITS (if any)
BCDP, PRI, NCI-FCRDC (T. Kamata)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.2	1.8

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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.)

Xenopus oocytes have become an ideal system for studies of signal transduction pathways, especially for the function studies of ras proteins. In order to use Xenopus oocytes as a model system, it is important to characterize the structures of Xenopus proteins in which we are interested in our own studies. Previously, the cDNAs encoding for protein kinase C (PKC), PI-specific phospholipase C (PLC), and various G-proteins have been molecularly cloned from the mammalian species. Using the mammalian cDNAs as the probes, Xenopus laevis cDNA clones, encoding for Goα, Giα, ADP-ribosylation factor (ARF), ras, two types of PLC, and two types of PKC have been isolated from Xenopus oocytes in our laboratory. These cDNA clones have been sequenced, and 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 suggests that the proteins are functionally interchangeable among different biological systems. Because Xenopus is evolutionarily closer to mammalian species than yeast, the Xenopus oocyte system would be a better choice for the functional studies of mammalian proteins in signal transduction. We have purified mammalian ras, ras GTPase activating protein (GAP), ARF, and PKC, and studied their functions in Xenopus oocytes. Our findings are summarized as follows: 1. GAP induces a rapid increase of diacylglycerol (DG) and GAP action is downstream or independent of ras functions in the stimulation of DG formation; 2. ARF has an antagonistic effect on ras actions; and 3. PKC potentiates ras- and insulin-induced oocyte maturation and ribosomal protein S6 phosphylation. Although our research efforts have been focused on ras-associated functions in Xenopus oocytes, we have also studied the regulation of oocyte maturation by other biological response modifiers (BRMs). For example, we have shown the negative regulation of progesterone-induced oocyte maturation by prostaglandin E1 or E2. Biochemical mechanisms of the inhibitory effects are currently under investigation.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LPB, NCI
Jian Zhao	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 $G_{o\alpha}$, $G_{i\alpha}$, ADP-ribosylation factor (ARF), ras, two types of phospholipase C, and two species of PKC from the Xenopus oocytes cDNA library. Because of the conserved nature of these proteins, we have studied the functions of mammalian proteins involved in signal transduction using Xenopus oocytes as the model system.

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. Also, we have studied the regulation of oocyte maturation by other BRMs.

METHODOLOGY

The following techniques were used: 1. molecular cloning; 2. DNA sequencing; 3. Southern and Northern blottings; 4. nuclease S1 mapping; and 5. gene expression.

MAJOR FINDINGS

Our molecular cloning studies indicate that the proteins involved in signal transduction are very conserved between Xenopus and mammalian species. These include $G_{o\alpha}$, $G_{i\alpha}$, ARF, ras, PLC, and PKC.

Purified mammalian ras, ras GTPase activating protein (GAP), ARF, and PKC were used for the functional studies of these proteins in Xenopus oocytes. The results are summarized below: 1. microinjection of GAP into oocytes rapidly induced an increase in the level of DG, similar to that observed upon microinjection of oncogenic ras protein. DG appeared to be derived mainly from the hydrolysis of phosphatidylcholine. Unlike ras protein, GAP did not induce

oocyte maturation. These results suggest that to induce full biological responses, ras proteins produce another signal(s), in addition to the one leading to DG formation through GAP. Furthermore, coinjections of GAP with neutralizing anti-ras antibody did not inhibit the GAP-induced increase of DG production suggesting that GAP action is downstream or independent of ras functions in the stimulation of DG formation. Our studies provide a new biological activity for GAP involving phospholipid metabolism and potentially a novel means of regulating phospholipid dependent protein kinase. 2. The physiological functions of ARF have been investigated in Xenopus oocyte system. Our results demonstrate the antagonistic effect on ras- and insulin-induced oocyte maturation. 3. We have shown that microinjected PKC potentiates ras protein action by increasing the rate of induction of both oocyte maturation and ribosomal S6 protein phosphorylation. The synergistic effect of PKC on ras- induced S6 phosphorylation is due to an overall increase of S6 derivatives, rather than a qualitative change in species of phosphorylated S6 derivatives. Our data further emphasizes the regulatory role of ras proteins and PKC in signal transduction in early development.

In addition to the studies of intracellular events involved in signal transduction in Xenopus oocytes, we have also studied the effects of oocyte maturation by external regulators. One class of metabolic regulators deserving special attention is the prostaglandins (cyclic derivatives of 20-carbon fatty acids). Prostaglandins are produced in most tissues and, hence, affect almost every organ system. Since the maturation of Xenopus laevis oocytes can be easily determined, we have used this system to study the effects of prostaglandins (PG) on oocyte maturation. Although, PGE and PGE_2 could not trigger Xenopus oocytes to undergo meiosis, they inhibited the rate of progesterone-induced oocyte maturation. Biochemical mechanisms of the inhibitory effects are currently under investigation.

FUTURE PLANS

Our future plans include the following: 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 antisense 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; 5. to investigate the biochemical mechanisms of ARF actions; 6. to identify the phospholipase(s) responsible for GAP actions; 7. to investigate the biochemical mechanics of the inhibitory effects by PGE on progesterone-induced oocyte maturation; and 8. to study the regulation of oocyte maturation by various BRMs.

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 1989;12(586):251-60.

Peng Z, Calvert I, Clark J, Helman L, Kahn R, Kung HF. Molecular cloning, sequence analysis, and mRNA expression of human ADP-ribosylation factor, Biofactors 1989;2:45-9.

Kamata T, Kung HF. Modulation of maturation and ribosomal protein S6 phosphorylation in Xenopus oocytes by microinjection of oncogenic ras protein and protein kinase C, Mol Cell Biol 1990;10:880-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09318-02 LBP

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Control of Cytokine/Cytokine Receptor and HIV 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
	I. Calvert	Chemist	LBP, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI, NCI-FCRDC (Raziuddin, T. Tan, Y. Huang)

LAB/BRANCH
Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	0.5	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.)*

The BRMP has a keen interest in cytokine and AIDS research, therefore, the LBP has initiated a study on the identification of sequence-specific DNA-binding proteins regulating the transcription of IL-2/IL-2R promoters and HIV-LTR. Using a Southwestern screening technique, several cDNA clones encoding the proteins that bind to different DNA elements have been isolated. One group of the mouse cDNA clones encoding the proteins that bind to the positive regulatory element of the IL-2R promotor sequence -261 to -244. The deduced amino acid sequence contains a zinc-finger DNA binding motif ($C\text{-}X_3\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_6\text{-}C$), followed immediately by a short leucine zipper structure ($L\text{-}X_6\text{-}L\text{-}X_6\text{-}L$), characteristic of DNA-binding proteins. A second mouse cDNA clone encodes the protein that binds to the sequence -94 to -64 of the IL-2 promoter. It has been reported that this DNA element is involved in the negative regulation of IL-2 gene expression in resting T-cells. Functional roles of these DNA-binding proteins in determining T-cell specific gene expression of IL-2/IL-2R will be investigated.

Both the IL2Rα gene and the HIV-LTR are activated by various T cell mitogens. Therefore, we have extended our IL-2/IL-2R studies to the HIV system. A 12 bp sequence present in the regulatory region of the IL-2Rα gene (sequence -267 to -256) is remarkably similar to the HIV enhancer element (HIV-DR; DR: direct repeats). Using HIV-1 enhancer oligonucleotides (sequence -137 to -17) as the probe, we have isolated a human cDNA clone by Southwestern screening technique. Characterization of the cDNA clone is in progress.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Jian Zhao	IRTA Fellow	LBP, NCI
Ida Calvert	Chemist	LBP, NCI

INTRODUCTION

T-cells mediate important regulatory functions in the immune response. The activation of T-cells involves both the production of IL-2 and the display of IL-2 receptors (IL-2R). It has been reported that HIV production from latently infected T lymphocytes can be induced with compounds that activate the cells to secrete lymphokines. Regulation of gene expression primarily operates at the level of transcription, and one way for the control of transcription is achieved through the interaction of trans-acting factors with the template DNA elements. For the studies of the transcriptional control of IL-2/IL-2R and HIV genes, we have initiated molecular cloning of the cDNAs encoding for the sequence-specific DNA binding proteins.

OBJECTIVES

The goal of this project is to investigate the transcriptional control of cellular (cytokines and cytokine receptors) and viral (HIV) gene expression during cell differentiation/proliferation and during infection/transformation by human retroviruses (HIV). The results of this study should provide fundamental information regarding eukaryotic gene expression and host-virus interactions that lead to the development of AIDS. 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

The following techniques were used: 1. preparation of nuclear extracts 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; and 7. cDNA library screening and DNA sequencing.

MAJOR FINDINGS

Using a Southwestern screening technique, three groups of cDNA clones encoding the proteins that bind to the specific elements of IL-2/IL-2R promoters and HIV-LTR have been isolated and characterized. The results are summarized as follows: 1. Two similar mouse cDNA clones encoding the proteins that bind to the positive regulatory element of the IL-2R promoter (sequence -261 to -244). The insert sizes for these two cDNA clones are 3.3 kb and 2.0 kb. DNA sequence-specific binding to the IL-2R promoter element was demonstrated by the gel retardation assays combined with competition analysis. Southern blot analysis indicated that

a single mouse gene encoded this binding protein. Northern blot analysis showed a major transcript at ~3.4 kb. The deduced amino acid sequence contains a zinc-finger DNA binding motif ($C\text{-}X_3\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_6\text{-}C$), followed by a short leucine zipper structure ($L\text{-}X_6\text{-}L\text{-}X_6\text{-}L$) characteristic of DNA-binding proteins. 2. Another mouse cDNA clone encoding the protein that binds to IL-2 regulatory element (sequence -94 to -64) has been isolated. It contains an insert of ~5.2 kb in size. Sequence data indicated that this is a newly discovered gene. The clone will be further characterized. 3. A human cDNA clone encoding the protein that bind to the HIV enhancer element (sequence -137 to -17) has been isolated. The gel-retardation assays combined with competition analysis showed the sequence specificity of DNA binding. Sequence analysis of the cDNA clone is in progress.

FUTURE PLANS

1. To purify the cloned DNA-binding proteins and to prepare antibodies against these proteins for the functional studies of these factors in the in vitro transcription systems and gel retardation assays.

2. Transfection assays will be used for the functional studies of the cloned cDNAs.

3. To isolate human counterparts of the mouse cDNA clones.

4. If homologous DNA binding proteins are expressed in Xenopus, functional studies will also be investigated in the Xenopus oocyte system.
5. Further analysis of the tissue distribution of these DNA-binding proteins.

PUBLICATIONS

Mikovits JA, Raziuddin, Gonda M, Ruta M, Lohrey NC, Kung HF, Ruscetti FW. Negative regulation of human immune deficiency virus replication in monocytes, J Exp Med 1990;171:1705-20.

SUMMARY REPORT

CLINICAL RESEARCH BRANCH

October 1, 1989 through September 30, 1990

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 building houses not only the outpatient clinic of the CRB, but also a radiotherapy suite and offices of private local oncologists. The CRB occupies approximately 12,000 square feet which includes a four-patient blood drawing area, clinical laboratory, nursing station and waiting area, four examining rooms, a large treatment area, and an eight-bed day hospital. Adjacent to the patient care area is an office suite for branch physicians, secretaries, data management personnel, and administrative staff.

The CRB research efforts concentrate on the evaluation of biological response modifiers (BRM). This includes phase Ia and Ib clinical trials to determine the spectrum of toxicity and immunomodulatory properties of BRMs and phase II therapeutic trials in specific disease types using the previously determined maximum-tolerated dose or optimal immunomodulatory dose. 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.

In the past year, the CRB has continued to investigate the potential role of IL-2 either alone or in combination with chemotherapy, other biologicals or effector cells in the management of patients with cancer. We recently published the results of an early BRMP trial describing the immunomodulatory properties and toxicity of chronic administration of low doses of IL-2. Other protocols have established that twice-weekly administration of high dose IL-2 by 24-hour continuous infusion is tolerable and very effective at inducing large numbers of lymphocytes in the peripheral blood with LAK activity. Large numbers of these cells with significant LAK activity could be maintained by further twice-weekly IL-2 at much lower doses. Using these methods, endogenous LAK cells could be induced and maintained for months. Unfortunately the presence of these cells alone was insufficient to result in significant numbers of antitumor responses. Attempts to improve this regimen have included the addition of cyclophosphamide, poly ICLC, flavone acetic acid (FAA) and a monoclonal antibody specific for melanoma called R24.

The addition of cyclophosphamide did little to change the toxicity or immunomodulatory properties of IL-2 and did not result in increased tumor responses. Poly ICLC was added to this regimen to determine the immunomodulatory properties of this agent alone and in combination with IL-2. Poly ICLC at the doses employed had no immunomodulatory properties and apparently had no effect on IL-2 induced changes. There were no tumor regressions. FAA was added to IL-2 because of the excellent results seen in animal tumor models when both agents are given together. This study included short periods of treatment with FAA alone to determine its toxicity and to examine its immunomodulatory properties before beginning combination treatment with IL-2. FAA was given at eight different doses by three different infusions (1, 3 or 6 hours). Patients on the early portion of the trial had their urine alkalinized, but patients on the second portion did not because of animal data suggesting that alkalinization may abrogate the therapeutic effects of IL-2 plus FAA. The toxicities of treatment with FAA were elucidated, but we were unable to detect any immunologic changes in patients following treatment with FAA, regardless of the dose or duration of the infusion. The only immunologic changes were noted during IL-2 therapy and were typical of those described in other trials. No tumor responses have been observed in 36 patients treated to date. Neither the immunologic nor therapeutic effects described in mice have been duplicated in cancer patients.

Since large numbers of activated killer cells with Fc receptors on their surface are generated during IL-2 therapy, the monoclonal antibody R24 was added to the twice-weekly regimen. R24 recognizes a ganglioside called GD3 that is present on melanoma cells and a subpopulation of T cells. R24 has been shown to exert antitumor effects itself and was chosen for that reason as well as for the potential to mediate antibody-dependent cellular cytotoxicity (ADCC) with the cells generated by IL-2 and because of its ability to directly activate T cells via GD3. Ten patients have been enrolled and there have been one mixed response and four partial remissions in 9 evaluable patients.

IL-2 has also been combined with IFN-alfa in a phase I study employing subcutaneous outpatient administration and with cisplatin and IFN-alfa in a phase II trial in patients with melanoma. The latter study included high-dose cisplatin on d1 and d8 (100 mg/m^2 in the first 4 patients and 75 mg/m^2 in the next 5) alternating with subcutaneous IFN-alfa 5 mu/m^2 given concomitantly with continuous infusion IL-2 at 3 $mu/m^2/d$. There were three partial remissions in 8 evaluable patients but there was severe gastrointestinal, hematologic and renal toxicity.

Trials of adoptive immunotherapy with IL-2 given in combination with LAK cells continued. A response rate of 20% was noted in patients with renal cell cancer (8/40) and melanoma (8/40) after treatment with continuous infusion IL-2, LAK cells, low-dose cyclophosphamide and adriamycin followed by IFN-alfa. Treatment at higher doses of IL-2 was associated with dose-limiting pulmonary toxicity when LAK cells were administered. The addition of immunomodulatory doses of chemotherapy and IFN-alfa to LAK and IL-2 did not improve response rates in patients with melanoma and renal cell cancer. The current trial of adoptive immunotherapy includes IL-2, LAK cells and the monoclonal antibody R24 in patients with melanoma.

BRMP studies have previously determined the immunologically active doses of IFN-gamma in patients with melanoma treated in the adjuvant setting. A study

performed during the past year found that comparable immunologic effects could be elicited at the same dose of IFN-gamma in patients with metastatic disease. IFN-gamma at 100 mcg or 250 mcg/m^2 resulted in similar immunologic effects. Since 250 mcg/m^2 is very close to the MTD for chronic therapy with IFN-gamma, and the response rate of IFN-gamma at the MTD is only 11%, it was determined that the immunologic effects were not sufficiently different to warrant therapy of patients at the 100 mcg/m^2 dose to determine the efficacy of treatment at a biologically effective dose.

The recent success of 5FU and levamisole in reducing recurrence rates and improving survival for patients with Duke's C colon cancer has led to renewed interest in the antiparasitic compound levamisole. One mechanism by which it may exert its effects is via the immune system. Levamisole alone was given every other day for six doses, followed by a two week washout period and then restarted in combination with IFN-gamma. The two arms of the study included one with patients with metastatic disease and another with adjuvant therapy for patients with melanoma or renal cell carcinoma. Forty patients have been treated (20 on each arm). Dose-limiting toxicity was observed at the 10 mg/m^2 dose of levamisole. Immunologic results are currently being analyzed.

Interleukin-1 has great potential as a bone marrow protective or restorative agent as well as an antitumor agent in its own right. Before complicated trials can be designed to examine these effects, phase I studies determining the toxicity and immunomodulatory properties must be performed. The first phase I trial of IL-1 alfa was performed at the BRMP with 28 patients receiving five different doses of IL-1 alfa with or without indomethacin. The MTD for IL-1 alfa alone was 0.3 mcg/kg and for IL-1 alfa and indomethacin it was 0.1 mcg/kg. Hypotension and renal insufficiency were the dose-limiting toxicities. Dose-related increases in peripheral blood granulocyte counts were noted. These findings have permitted us to initiate a phase II trial of IL-1 alfa plus indomethacin at the MTD in patients with melanoma and an additional trial in patients with non-small cell lung cancer to examine the myeloprotective or myelorestorative properties of IL-1 alfa given before or after high-dose (800 mg/m^2) carboplatin. A phase I trial of IL-1 beta is currently in progress.

Antibodies to the CD3 determinant on peripheral blood T cells may be useful in the immunotherapy of cancer. Crosslinking the CD3 determinant on malignant proliferating T cells with monoclonal antibody results in growth inhibition in vitro. This BRMP laboratory observation led to the design of a phase I study in patients with CD3-positive malignancies. Three patients with mycosis fungoides have been treated with anti-CD3. There were no responses and one patient developed a severe headache.

Anti-CD3 antibodies also have the capacity to activate T cells to proliferate, secrete lymphokines and to become cytotoxic (either non-specifically or specifically) to tumor cells. We conducted a phase I study of anti-CD3 in patients with a variety of solid tumors. Patients received four doses of antibody by three-hour infusion over two weeks. Dose-limiting toxicity occurred in all three patients receiving 100 mcg and the MTD was determined to be 30 mcg. Dose-limiting toxicity consisted of severe headache associated with fever, photophobia and a stiff neck. The toxicity resembled aseptic meningitis and was accompanied by the following abnormalities in the cerebrospinal fluid: increased pressure, pleocytosis of predominantly mature lymphocytes and elevated protein.

An additional five patients received 30 mcg by i.v. bolus to see if the infusion schedule affected toxicity; severe headache was noted in the majority of these patients as well. Additional patients were treated with 14 daily injections of low dose anti-CD3 and severe headache was observed. No dose-related immunologic changes were noted in patients receiving anti-CD3 in this study.

The severe toxicity associated with anti-CD3 has led us to seek alternate methods of using this agent. Patients are now accruing to a protocol in which they are pheresed and their peripheral blood lymphocytes are activated with anti-CD3 in vitro for 16-24 hours. Following in vitro activation, cells are washed and adoptively transferred to the host in combination with systemic IL-2 therapy.

A number of protocols employing granulocyte macrophage-colony stimulating factors (GM-CSF) are currently being performed by the CRB. A phase Ib trial of intraperitoneal administration of GM-CSF is being performed to increase the number of peritoneal monocytes/macrophages and then to assess their state of activation before and after further therapy with intraperitoneal IFN-gamma or IL-2. Two patients have been enrolled in this study. Other studies are investigating the ability of GM-CSF to enhance the delivery of chemotherapeutic drugs. In a trial performed in collaboration with the Medicine Branch, Clinical Oncology Program, NCI, patients with refractory ovarian cancer received GM-CSF to determine its effects on myelosuppression associated with carboplatin (800 mg/m^2) given every 35 days. Eight patients were treated at the BRMP and all eight experienced significant myelosuppression, requiring dose reductions in seven. All patients experienced severe thrombocytopenia and required platelet transfusions. Three responses were seen in the eight patients. Granulocytopenia may have been affected favorably, but thrombocytopenia was not ameliorated and became the dose-limiting toxicity. GM-CSF is also part of a protocol designed to increase the dose intensity of MOPP chemotherapy for poor prognosis patients with Hodgkin's disease. To date, five of six evaluable patients have achieved a complete response.

An additional trial has been designed to take advantage of the synergistic antiproliferative effects of IFN-alfa and poly ICLC. To date, 17 patients have been entered and there have been two minor responses and one partial response. A unique combination of agents (5FU, leucovorin, azidothymidine (AZT) and persantine; FLAP) has been used in patients with melanoma and colorectal or renal cell cancer. A phase I portion of the study determined the tolerable doses of AZT and a phase II portion of the study is in progress to examine the efficacy of this regimen. Monitoring of proliferation of peripheral blood lymphocytes will be performed to determine how effective thymidine starvation was at the cellular level.

The CRB continues to perform a number of disease-oriented protocols. A large number of patients with hairy cell leukemia continue to be followed on treatment with IFN-alfa or after completion of therapy with alternating cycles of IFN-alfa and deoxycoformycin. Of the former, 32 patients continue to receive IFN without interruption for a median of 60 months. Twenty-one patients discontinued IFN for a variety of reasons, the most common being the development of acquired resistance after the formation of neutralizing antibodies. These neutralizing antibodies neutralize only IFN alfa-2a suggesting that alternative non-cross reactive species of IFN may be used in these patients. Further follow-up of patients with neutralizing antibodies without resistance to IFN and of patients with non-neutralizing antibodies gave surprising results. Many patients that

had previously had either non-neutralizing or neutralizing antibodies became antibody negative and no patients who were antibody negative became antibody positive. There have been no definite late interferon-related toxicities but two patients developed an absolute erythrocytosis late in the course of therapy leading to discontinuation of IFN in one.

Fifteen patients continue to be followed for a median of 35 months after discontinuation of IFN and deoxycoformycin. All patients continue to have normal peripheral blood counts with no circulating hairy cells and bone marrows that contain <5% hairy cells. Although the initial response rate may not be different than deoxycoformycin alone, the duration of the responses are remarkable, particularly in comparison to IFN-treated patients. Marked depression in T cell number and function was found in these patients but other than an unusually high incidence of localized Herpes zoster infections, there were no opportunistic infections. With time off treatment, the number of T cells is returning to normal in the majority of patients. We continue to enroll patients with hairy cell leukemia who fail IFN therapy on a salvage protocol using deoxycoformycin. Patients with T-gamma lymphoproliferative disorder who are IFN resistant are also eligible for this protocol.

The CRB also maintains clinical protocols for the management of patients with non-Hodgkin's lymphomas and Hodgkin's disease. Patients with aggressive lymphomas will be treated according to their stage. Stage I patients are treated with four cycles of ProMACE-MOPP chemotherapy at 75% doses followed by involved field radiation to 40 Gy. There are 55 evaluable patients entered on study of whom 53 (96%) achieved a complete remission. There has been one relapse and only three deaths. Advanced stage patients are treated with short course ProMACE-CytaBOM which 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. To date, there are 23 evaluable patients; 19 have achieved CRs (83%). There have been 6 relapses (32%). Early response rates are equivalent to standard ProMACE-CytaBOM. Standard ProMACE-CytaBOM is still employed to treat patients with angiocentric immunoproliferative lesions. To date, all four patients have achieved a CR and there was one relapse who was salvaged with high-dose chemotherapy and autologous bone marrow transplant.

Patients with low grade or indolent lymphomas are randomized to observation with delayed therapy if symptoms occur or to intensive combination chemotherapy with ProMACE-MOPP flexitherapy and modified total nodal irradiation. There are no significant differences in disease-free or overall survival, although there are differences in complete response rates between the two groups with 75% of patients randomized to intensive therapy achieving a CR compared to 29% for patients treated after initial observation. The median follow-up is six years; this is still shorter than the median survival (10 years) of patients with these diseases.

Patients with advanced stage Hodgkin's disease are treated with dose intense MOPP and GM-CSF (described earlier) if they have a poor prognosis (IIIB, IVB, IVA with bone marrow involvement) or MOPP if they have IIIA or IVA without marrow involvement. Patients with early stage Hodgkin's disease are randomized to MOPP chemotherapy or radiation. Of the 54 evaluable patients randomized to MOPP, 52 (96%) achieved a complete response; 7 relapsed. Forty-nine of 51 patients treated with radiation achieved a CR (96%); 17 relapsed. The projected 10-year disease-free survival for radiation-treated patients is 60% and 86% for MOPP-

treated patients ($p_2 = 0.009$). Overall 10-year survival is 76% for radiation and 92% for MOPP ($p_2 = 0.051$). This modest survival benefit is lost if patients with stage IIIA disease and massive mediastinal disease are excluded. Long-term toxicity information is being collected.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09291-05 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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:	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, 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-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	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.)*

We administered deoxycoformycin (dCF) and interferon-alfa sequentially to patients with hairy cell leukemia in an attempt to improve the response rate and duration observed in earlier studies using each drug alone. Further rationale for this study included the observation that interferon-alfa resistant patients were nearly uniformally responsive to second line dCF. As there was some overlapping toxicitiy, we decided to administer the drugs sequentially rather than concurrently. In this study, we evaluated patients for response by performing bilateral iliac crest bone marrow biopsies and aspirates since the peripheral blood normalizes rapidly after either interferon-alfa or dCF treatment but disease becomes patchy in the bone marrow and might be missed if only unilateral bone marrow biopsies had been performed. Other studies using interferon-alfa or dCF alone used unilateral marrow examinations in evaluating response. Using these more relaxed criteria of response, authors had published high rates of complete remission to dCF. Of 15 patients entered in our study, 14 are evaluable for response with one patient having diffuse osteosclerosis not being evaluable for marrow response. All patients had rapid normalization of peripheral blood counts and all 14 marrow-evaluable patients were found to have very small numbers (less than 5% of the total marrow cellularity) of residual hairy cells at the completion of treatment. With a median follow-up now of 35 months, no patient has had an increase in the number of hairy cells in the bone marrow or any change in the peripheral blood. Although the initial response rate to the combination of dCF and interferon appears to be no better than that observed with dCF alone, the response duration may be longer. These patients continue to be followed for determination of response duration.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
John E. Janik	Expert	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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 interferon alfa.

4. To determine the biochemical consequences of administering deoxycoformycin with a biological response modifier.

METHODS EMPLOYED

Patients 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 interferon alfa 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 received 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 determined 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 35 months 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 toxicities of this therapy consisted of nausea and vomiting in thirteen patients, depression in eight, paresthesias in five, dermatitis in ten, minor eye irritation without corresponding clinical signs in six and dermatomal herpes zoster infections in five. One patient with normal peripheral blood counts and stable minimal hairy cell infiltrates in the bone marrow died after an inadvertent over dose of a sleeping pill after completion of therapy. It was felt unlikely that this was related to therapy. The herpes zoster infections generally occurred late in therapy and may be indicative of an underlying suppression of immune function. Hematologic toxicity included progressive neutropenia early in the course of therapy in all 15 treated patients, 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/mm^3 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/mm^3 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 has been followed closely in these patients. As of May 1990, most patients continue to have marked suppression of CD4 counts. The clinical ramifications of this

suppression however have been minimal. Five patients have now had localized dermatomal zoster either during or after therapy. No patients have had more traditional 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, follow-up studies have shown that relapses are occurring. 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 interferon alfa 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

Martin A, Nerenstone S, Urba WJ, Longo DL, Lawrence JB, Clark JW, Hawkins MJ, Creekmore SP, Smith II JW, Steis RG. Treatment of hairy cell leukemia with alternating cycles of pentostatin and recombinant leukocyte A interferon: Results of a Phase II study, J Clin Oncol 1990;8:721-30.

VanderMolen LA, Urba WJ, Longo DL, Lawrence J, Gralnick H, Steis RG. Diffuse osteosclerosis in hairy cell leukemia, Blood 1989;74:2066-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09298-05 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the bordars.)*

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
	J. E. Janik	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (J. Hursey)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	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.)*

Interleukin-2 (IL-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 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 the infusion of lymphokine activated killer cells (LAK) 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 20% in melanoma (8/40) and 20% in renal cell cancer (8/40). The higher IL-2 dose resulted in substantial, dose-limiting pulmonary toxicity when LAK cells were administered. 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.

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
John E. Janik	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI

OBJECTIVES

To increase the antitumor activity of a regimen employing continuous infusion interleukin-2 (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 16 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

A total of 90 patients were accrued to the study. Eighty-five are evaluable for toxicity and 80 are evaluable for response. Among the 40 patients with metastatic melanoma, there were 8 partial responses (20%) with a median duration of 3.5 months. All responses occurred in lung, lymph node or skin (cutaneous or subcutaneous) with the exception of a single patient who achieved a partial response in a large pelvic mass. Two of the responders are currently free of disease (with residual scars detected by radiologic scans at previously involved sites) greater than 12+ months from initiation of therapy.

Eight of 40 renal cell carcinoma patients (20%) achieved partial responses. The median duration of response was 4.5 months. All responses occurred in lung, pleura, or lymph nodes with the exception of a single patient who had a partial

regression in an expanding bone lesion. The response rate in the subgroup of patients with disease limited to lung, lymph node, or pleura was 33%, and 3 of the patients had almost complete disappearance of all sites of disease.

Toxicity was well tolerated at the lower dose of IL-2 (3 mu/m^2/d). Only 4/29 (14%) required pressor support and no patient had serious pulmonary toxicity. At 6 mu/m^2/d, substantial toxicity was seen. Four patients died as a result of treatment (10%). Fifty percent had sufficient pulmonary compromise to require continuous oxygen supplementation. Other serious toxicities included hypotension requiring pressors (39%), intubation for respiratory failure (2%), bowel perforation (2%), pericarditis (2%), and adrenal hemorrhage (2%). Reversible toxicities included creatinine elevation, elevation of bilirubin and transaminases, mucositis, nausea, vomiting, and diarrhea, all of which were more severe at the higher IL-2 dose level.

Although no apparent dose-response was noted with respect to tumor regression, all durable responses (which also corresponded to patients achieving a near complete remission) were seen at the higher dose of IL-2 (6 mu/m^2/d). The addition of cyclophosphamide, doxorubicin, and interferon-alfa did not appear to improve the activity of the regimen when compared to the response rates for IL-2/LAK reported in the literature. However, other investigators have found that continuous infusion IL-2 (with LAK) is inactive in patients with melanoma; therefore immunomodulatory doses of chemotherapy and interferon-alfa may have contributed to the antitumor activity of this IL-2/LAK regimen.

PROPOSED COURSE

The study was temporarily closed after meeting its initial goals and reopened under a new principal investigator, Dr. John Smith II. A cohort of patients will be treated to study the trafficking of infused LAK cells. The effects on cell traffic by varying the length of in vitro culture and pretreatment of the patient with cyclophosphamide and doxorubicin prior to cell infusion will be studied.

PUBLICATION

Sznol M, Clark J, Smith J, Steis R, Urba W, VanderMolen L, Janik J, Sharfman B, Fenton B, Creekmore S, Kremers P, Conlon K, Hursey J, Beveridge J, Longo D. A Phase II study of IL-2/LAK in combination with chemotherapy and interferon-alfa in patients with metastatic melanoma and renal cell carcinoma (RCC). In: Leventhal B, ed. Proceedings of American Society of Clinical Oncology. Washington, DC: American Society of Clinical Oncology 1990;9:(Abst. 719)186.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09305-04 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BRMP, 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, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	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.)*

This study of deoxycoformycin (dCF) in patients with hairy cell leukemia was written as a salvage protocol for patients who would progress or not respond to interferon in other ongoing Clinical Research Branch protocols. We also wanted to test the efficacy of this drug in T-gamma lymphoproliferative disorder since dCF had been found to have activity in other indolent T-cell diseases. Nine patients have now been treated on this protocol, five with hairy cell leukemia and four with T-gamma lymphoproliferative disorder. All patients were resistant to interferon alpha treatments and had varying degrees of peripheral blood cytopenias. Of the five patients with hairy cell leukemia, there were three partial responses, one nonresponder and one patient who died of refractory thrombocytopenia and diffuse gastrointestinal bleeding after only two doses of dCF. Of the four patients with T-gamma lymphoproliferative disorder, two have had partial responses and two were non-responders. We conclude that dCF has activity in interferon resistant hairy cell leukemia and in some patients with T-gamma lymphoproliferative disorder.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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 (dCF) in patients with hairy cell leukemia or T-gamma lymphoproliferative disorder refractory to or intolerant of low-dose alfa interferon.

2. Determine the biochemical consequences of the administration of dCF in patients with hairy cell leukemia or T-gamma lymphoproliferative disorder.

3. To determine the immunologic consequences of the administration of dCF in patients with hairy cell leukemia or T-gamma lymphoproliferative disorder.

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 alfa interferon therapy.

Once patients are deemed eligible for the study, they receive 4 mg/m^2 of dCF intravenously every week for three consecutive weeks. Patients then receive 4 mg/m^2 on alternating weeks. Patients 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. dCF is administered after hydration with 1 liter of D5 and half normal saline. After administration of the dCF, one further liter of fluid is given intravenously.

MAJOR FINDINGS

Five patients with interferon resistant hairy cell leukemia have been treated and three partial responses were obtained. One patient did not respond and one patient died of refractory thrombocytopenia and gastrointestinal bleeding after two doses of dCF. This rate of response (60%) is lower than that reported by other groups treating interferon-resistant hairy cell leukemia with dCF. The explanation for this is likely that other groups define interferon resistance as recurrence of disease following discontinuation of interferon after 12 to 18 months of therapy. Our patients in contrast were required to show either no response or disease progression during interferon therapy. Thus, truly interferon-resistant hairy cell leukemia patients probably have a lower response to dCF than is currently evident from the literature.

Four patients with interferon-resistant T-gamma lymphoproliferative disorder have been treated and two partial responses have been observed. The two responding patients were elderly women with a long-standing history of granulocytopenia and anemia. One patient had recurrent infections. Both patients normalized peripheral blood counts after treatment with dCF and one patient has been off therapy for over two years and continues to have normal peripheral blood counts. She however continues to have moderate numbers of large granular lymphocytes in the bone marrow that appear to be causing no detrimental effects at least on peripheral blood counts. The other responding patient has just recently completed therapy.

SIGNIFICANCE

Interferon therapy is not curative for hairy cell leukemia and patients initially responding to this drug are at continuing risk for disease progression. The development of treatment strategies for patients who have disease progression after discontinuation of interferon therefore will likely have applicability to the majority of patients who are initially responsive to interferon. Other treatment options include re-administration of interferon or use of other drugs such as fludarabine or 2'-chlorodeoxyadenosine. Our results confirm previous studies done at the Biological Response Modifiers Program and elsewhere that dCF is an effective salvage therapy for interferon-resistant hairy cell leukemia. The three responding patients on this study have continued in partial remission for a median of 30 months from the completion of dCF therapy.

The optimal management of patients with T-gamma lymphoproliferative disorder is unknown. Although corticosteroids, cytotoxic drugs, and splenectomy have been successfully used in small numbers of patients with T-gamma lymphoproliferative disorder, the rarity of this disease has not made it possible to adequately study any single treatment. We opted to use dCF in this setting because of its activity in other indolent T-cell diseases such as mycosis fungoides and T-cell ALL. Unfortunately, accrual to this study has been slow but substantial responses have been observed in at least half of our patients so far. We obviously will need to treat more patients before definitive recommendations for the use of dCF in this disease can be made.

PROPOSED COURSE

The protocol is being kept open for further patient accrual should interferon resistance ultimately develop in more of our hairy cell patients. We also hope to accrue more patients with T-gamma lymphoproliferative disorder.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09306-04 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. E. Janik	Expert	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, 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, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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.)*

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 Biological Response Modifiers Program 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.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
John E. Janik	Expert	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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 traffic 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 09308-04 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	J. E. Janik	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	3.5	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.)*

This study began as an efficacy study of interferon alfa-2a in patients with hairy cell leukemia. It was observed that most patients responded to interferon, but that very few complete responses were being obtained. Studies being done elsewhere confirmed the low complete remission rate. Once interferon was stopped, nearly uniformly disease progression requiring reinstitution of therapy was observed. There appear to be very few if any patients who will not require further therapy after 12 or 18 months of continuous interferon treatment. Because of these findings, we opted to administer interferon continuously to patients who were initially responsive to this drug. Of the 53 evaluable patients (of the 56 entered on this study), there was one complete remission, 34 partial remissions, 3 minor responses, 9 patients with stable disease and only 1 patient with disease progression. Thirty-two patients continue to receive interferon without interruption with a median duration of continuous interferon treatment of 60 months. Twenty-one patients discontinued interferon for a variety of reasons the most common of which being the development of acquired interferon resistance in association with interferon antibodies. Although it is clear from this study that the disease can be controlled in the long-term with interferon, longer follow-up will be necessary to determine if this form of therapy is better than intermittent therapy with interferon.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Robert G. Fenton	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
John E. Janik	Expert	CRB, 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. Initial responses included one complete remission, 39 partial remissions, three minor responses, nine patients with stable disease, and one patient with disease progression. There was no difference in response rate between previously splenectomized patients (30 patients) and those who had not previously undergone splenectomy (23 patients). The therapy continues to be well tolerated.

Twenty-one patients have been removed from therapy because of failure to respond initially (1) disease progression after initial response (7), myocardial infarction (1), squamous cell carcinoma of the lung (2), erythrocytosis (1), noncompliance (1), patient refusal (1), depression (1), impaired cognition (2), fatigue (1), sensory neuropathy (1), recurrent CVA's (1) and flare of rheumatic

syndrome (1). The single most common cause for removal from therapy, therefore, was disease progression following initial responsiveness. This acquired interferon resistance was found to be related to the development of neutralizing interferon antibodies; we have not observed acquired interferon resistance in the absence of these antibodies. The neutralizing antibodies found in our patients neutralized only interferon alfa-2a; when sera were tested for their neutralizing ability against a preparation of partially purified natural interferon, no neutralizing activity was found. This suggests that if patients develop antibody-mediated interferon resistance, alternative non-cross reactive species of interferon may be used effectively.

It appears, therefore, that one important issue in the long-term management of patients with interferon is the development of neutralizing interferon antibodies. Because of the importance of this phenomenon, we performed a follow-up study to evaluate the prevalence of neutralizing antibodies late in the treatment course and the effect these antibodies might have on biochemical and febrile responses induced by interferon. To our surprise, many of the patients who previously had either non-neutralizing or neutralizing antibodies became antibody negative and no patients who had been antibody negative previously became antibody positive. Of 10 patients who previously had neutralizing antibodies, three became antibody negative and five still had antibody but without neutralizing activity. Of nine patients who previously had non-neutralizing antibodies, all nine became antibody negative. These patients became antibody negative despite the fact that they continued to be treated with interferon alfa-2a. We only had one patient who had a neutralizing antibody against interferon alfa-2a at the time of this follow-up study. The functional studies revealed that non-neutralizing antibody had no effect on serum interferon levels, the febrile response to interferon, or the ability of interferon alfa-2a to increase serum levels of neopterin and cell-associated 2′, 5′oligoadenylate synthetase. The one patient who continues to have a neutralizing antibody had no detectable serum levels of interferon following subcutaneous administration of up to 9 million units of interferon alfa-2a. Further, there was no induction of neopterin or 2′, 5′oligoadenylate synthetase activity following injection of interferon alfa-2a. Only a slight fever developed in this patient. He had, however, the expected increase in 2′,5′oligoadenylate synthetase activity, neopterin levels, interferon levels, and temperature following administration of 9 million units of human lymphoblastoid interferon. These data are consistent with the notion that neutralizing antibodies completely inhibit the effects of interferon alfa-2a. The in vivo effects thus mirror the in vitro antibody specificity. That patients with neutralizing antibodies still remain responsive to interferon, however, can be inferred from the normal responsiveness to human lymphoblastoid interferon.

There have been no definite late interferon-related toxicities. Two patients developed an absolute erythrocytosis late in the course of therapy that resulted in discontinuation of interferon in one. This patient went on several months later to develop a small bowel infarction and died. Other than these two patients, however, no definite treatment-related toxic effects have been observed in the long-term.

As of this date, 32 patients continue to receive interferon alfa; 31 receive therapy at the standard dose of 3 million units 3 times weekly and one patient required dose escalation back to 3 million units daily to maintain the initial response he achieved.

SIGNIFICANCE

In this study we have therefore confirmed interferon's efficacy 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 attainment 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 attainment of an initial response to determine the optimal long-term management of these patients. At least it appears that the expected increased incidence of neutralizing interferon antibodies has not been observed and in fact antibodies have resolved despite continued administration of the drug. An adverse effect of these antibodies on our long-term ability to treat these patients, therefore seems unlikely.

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, and the development of interferon resistance to help determine whether chronic maintenance therapy with interferon is the most appropriate form of therapy to administer following an initial induction course.

PUBLICATIONS

Steis RG, VanderMolen LA, Lawrence J, Sing G, Ruscetti F, Smith II JW, Urba WJ, Clark J, Longo DL. Erythrocytosis in hairy cell leukaemia following therapy with interferon alpha. Br J Haematol 1990;75:133-35.

Steis RG, Smith II JW, Ewel C, Urba WJ, Barney R, Longo DL. Loss of serum interferon-alpha 2A (IFN- 2A) antibodies (ABS) during continuous long-term interferon therapy of hairy cell leukemia (HCL). In: Leventhal B, ed. Proceedings of American Society of Clinical Oncology. Washington, DC: American Society of Clinical Oncology 1990;9:(Abst. 702)182.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09324-03 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. E. 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, BRMP, 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, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 investigates the antitumor and immunological effects of chronic IL-2 administration. 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. 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 circulating Leu19+ cells. At 3 months, tumor response is assessed. Modest antitumor effect was seen in the initial trial (1 PR, 1 minor response in 17 patients). Patients maintained high Leu19 levels and increases in circulating NK and LAK activity. Patients also demonstrated bimodal Leu19+ populations: CD16+/Leu19 dim and CD16-/Leu19 bright. 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. A follow-on trial employed moderate doses of cyclophosphamide monthly to reduce suppressor cell populations. This trial showed a comparable ability to maintain sustained immunological enhancement with IL-2. This trial also showed modest evidence of antitumor effect (2 PRs, 1 minor response in 17 patients). Full analysis of the immunological data is underway. A second follow-on study (#8908) is being performed using a monoclonal antibody (R-24) which mediates ADCC, with this IL-2 regimen, for melanoma.

PROJECT DESCRIPTION

PERSONNEL

Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Robert 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, 17 patients have been entered on study; 15 are evaluable for antitumor response (8 melanoma, 3 renal cell, 1 ovarian, 1 breast, 1 NML, and 1 colon cancer). The remaining two patients were taken off study after the 1 month period of induction treatment, at their own request. 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. Two partial responses (1 melanoma, 1 renal cell) and 1 minor response (melanoma) have been seen; an additional patient (melanoma) was showing response at 1 month of treatment, but was taken off treatment at his own request.

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 modest toxicity of the maintenance phase of this regimen would appear to permit the addition of other treatment modalities. Therefore, two follow-on trials have begun design around this regimen with the addition of monoclonal antibodies recognizing determinants on colon cancer and melanoma cells, (MAbs NRCO-4 and R-24, respectively). Accrual has begun to the melanoma trial (#8908), but the colon cancer trial (#8907) has been delayed due to problems in antibody availability.

PROPOSED COURSE

Related trials are 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 09325-03 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. E. Janik	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Frederick Memorial Hospital, Frederick, MD (K. Madera); Program Resources, Inc., Frederick, MD (W. J. Urba, J. Beveridge, R. Hornung); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol); Roger Williams Hospital, Providence, RI (J. Clark)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 was modified to infuse the FAA over shorter periods of time (1 and 3 hour intervals) and to delete urinary alkalization. In this modification, 12 patients were entered on trial (4 colon, 2 melanoma, 1 rectal, and 5 renal cell cancer). Of these, 2 still remain on treatment. The trial continues to define an MTD of FAA by 1-hour infusion once weekly, in combination with IL-2 at a fixed dose of 3x106 u/m2 twice weekly, in this regimen. No antitumor responses have been seen. In the patients treated with 3-hour FAA infusion, some enhancement of NK and LAK activity was seen during the period of combined FAA and IL-2 therapy, but not during the initial 3-week period during which they were treated with FAA alone. More detailed analysis of immune modulatory data is continuing, as patients are accrued to other FAA dosage levels.

PROJECT DESCRIPTION

PERSONNEL

Stephen Creekmore	Chief	BRB, NCI
Robert H. Wiltrout	Senior Investigator	LEI, NCI
Ira Green	Senior Investigator	BRB, 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, BRMP, NCI
John E. 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. In the first trial, FAA was given once weekly by a 6-hour infusion; in the second trial, FAA was given once weekly by 3- or 1-hour infusions. IL-2 dosage in the second trial was fixed at $3x10^6$ u/m^2.

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 were entered on study using the 6-hour FAA infusion. 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. Twelve patients have been treated using the 3-hour infusion regimen (4 colon, 2 melanoma, 1 rectal, 5 renal cell cancer). There was 1 PR (colon cancer). Currently 2 remain on treatment; 6 have been taken off due to PD; 3 were removed due to toxicity, defining an MTD for the 3-hour infusion; 1 patient ended the treatment period with stable disease. Immune studies are currently still being examined as patients are accrued to the 1-hour infusion, to determine any potentiation of IL-2 effects by FAA.

Toxicities attributed to the regimen include grades 1-3 hypotension, grade 1-2 myalgia and weakness, increased CPK and LDH, increased 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 FAA/IL-2 synergy, observed in a murine renal cell carcinoma model, is optimal at high peak FAA levels, and without urinary alkalization. Accrual is continuing to the modified FAA trial to explore this observation, in the hope of achieving the desired FAA/IL-2 synergy in humans. Further studies will be undertaken depending on these results. Alternative FAA analogues are also being considered.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09329-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

Recombinant GM-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:	J. E. Janik	Expert	CRB, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Treatment Evaluation Program, NCI, Bethesda, MD (M. Sznol); Medicine Branch, NCI, Bethesda, MD (E. Reed)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	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.)*

This trial was designed to test the ability of recombinant granulocyte macrophage-colony stimulating factor (rGM-CSF) to protect patients with refractory ovarian carcinoma from the bone marrow suppressive effects of high-dose carboplatinum. A previous study with high-dose carboplatinum demonstrated a response rate of 27% in patients with advanced ovarian carcinoma. Therapy was associated with severe and prolonged myelosuppression. In this trial, carboplatinum was administered every 35 days at a dose of 800 mg/m sq. rGM-CSF was given at various doses beginning three days after administration of carboplatinum. This study is being conducted jointly with the Medicine Branch of the National Cancer Institute. Eight patients have been treated at the Biological Response Modifiers Program. All patients experienced significant myelosuppression following high-dose carboplatinum and all but one patient required dose reductions in chemotherapy due to this myelosuppression. All of the patients experienced significant thrombocytopenia and required platelet transfusions. All of the patients required hospitalization during the course of therapy. Two of the eight patients treated at the highest dose level of GM-CSF did not require hospitalization for granulocytopenia and fever. Whether GM-CSF shortens the duration of granulocytopenia cannot be determined from this study. Thrombocytopenia has remained severe and prolonged and appears not to have been affected by GM-CSF. Six of the eight patients treated at the Biological Response Modifiers Program are evaluable for response. Three responses, two partial and one complete response were seen.

PROJECT DESCRIPTION

PERSONNEL

John E. Janik	Expert	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
William H. Sharfman	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI

OBJECTIVES

1. To determine if recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) alters the pattern of myelosuppression associated with high-dose carboplatinum (800 mg/m^2) in patients with refractory ovarian carcinoma.

2. To determine if the dose intensity and/or cumulative dosage of carboplatinum may be increased in association with rGM-CSF administration.

3. To compare the response rate of high-dose carboplatinum with rGM-CSF to historical controls treated without rGM-CSF.

4. To evaluate the toxic effects of high-dose carboplatinum 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 cisplatinum 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 creatinine levels less than 1.5. White blood count greater than 3,000 per cubic millimeter and platelet count greater than 50,000 per cubic millimeter and SGOT and SGPT levels less than 100 units per milliliter.

After an initial staging evaluation, patients are administered carboplatinum at a starting dose of 800 mg/m^2 every 35 days. rGM-CSF is given as a subcutaneous injection starting 72 hours after carboplatinum and is given for a minimum of 18 days or until the total white blood cell count is above 10,000. GM-CSF dose levels include 3, 10, 20, and 30 micrograms per kilogram per day. An additional cohort of patients is being treated at a dose of 5 micrograms per kilogram per day. Antitumor effects are evaluated after four cycles of high-dose carboplatinum and GM-CSF therapy.

MAJOR FINDINGS

Eight patients have been admitted to this study at the BRMP. One of these patients is too early for antitumor evaluation. Of the seven remaining patients, two achieved a partial response and one a complete response to therapy. One additional patient was not evaluable for antitumor response due to toxicities

experienced in the first cycle of therapy. The remaining patients had progressive disease. The most frequent side effect seen in patients treated with high-dose carboplatinum in conjunction with rGM-CSF has been thrombocytopenia. This thrombocytopenia has, in some patients, been refractory to random donor platelet administration and in several patients was refractory to HLA matched platelets. All of the patients experienced Grade IV platelet toxicity with platelet counts below 20,000. Several of the patients experienced signs of thrombocytopenia including petechiae, hematuria, epistaxis and gastrointestinal hemorrhages. Fever was universally seen in association with rGM-CSF administration and in all but two of the patients was associated with neutropenia requiring hospitalization and intravenous antibiotic therapy. One patient was removed from study due to hypotension which persisted even after a 50% dose reduction while receiving rGM-CSF. One patient who achieved a partial remission after three cycles of therapy received no further therapy because of the development of staphylococcal endocarditis. All patients but one required carboplatinum dose reductions due to prolonged thrombocytopenia on this protocol.

SIGNIFICANCE

GM-CSF as administered in this trial is not capable of preventing the neutropenia and thrombocytopenia expected after administration of high doses of carboplatinum. rGM-CSF can cause fevers while patients are neutropenic and necessitate their hospitalization and administration of intravenous antibiotics. It is possible that rGM-CSF will reduce the incidence and/or severity of neutropenia and its associated complications. It does not appear that GM-CSF reduces the need for hospitalization in this group of patients. GM-CSF appears to have no effect upon the severity or duration of thrombocytopenia and the need for intensive platelet transfusion support. It remains necessary to develop a platelet sparing regimen or/an alternative agent to deliver adequate doses of carboplatinum to this group of patients.

PROPOSED PLAN

We will continue to accrue patients on this study. Administration of GM-CSF on a twice daily schedule at one day following chemotherapy has been demonstrated to have platelet sparing effects in patients with ovarian carcinoma. A small cohort of patients will be entered with this modified regimen of GM-CSF following high-dose carboplatinum to determine whether GM-CSF has significant platelet sparing effects when administered by a modified regimen.

PUBLICATIONS

Reed E, Janik J, Bookman M, Rothenberg M, Smith II J, Ozols RF, VanderMolen L, Jacob J. High dose carboplatin and rGM-CSF (GM) in refractory ovarian carcinoma. Proc Am Soc Clin Oncol 1990;9:157, abst. 609.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09330-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one lina 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. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	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.)*

Recombinant interferon (IFN) gamma has previously been shown to activate monocytes in patients with malignant melanoma rendered disease-free with surgery. The purpose of this protocol was to test three different doses of recombinant IFN-gamma in patients with metastatic disease (high tumor burdens) to see if there was an optimal immunomodulatory dose in these patients.

We enrolled 18 patients on this study and treated 5 patients at each of the following three dose levels: 0.01 mg/m sq, 0.1 mg/m sq, and 0.25 mg/m sq. In general, therapy was well-tolerated and toxicities were mild. Serial blood sampling for evidence of monocyte activation showed that the two higher doses produced consistent increases in monocyte activation. Statistical analysis revealed no significant differences between these two doses and there was no evidence for any superiority of the 0.1 mg/m sq dose over the 0.25 mg/m sq dose.

This study demonstrates that patients with relatively high tumor burdens (patients with metastatic malignant melanoma) can have significant monocyte activation induced by IFN-gamma. In contrast to our previous study, in patients with relatively low tumor burdens, this current study did not show any superiority of the 0.1 mg/m sq dose over the 0.25 mg/m sq dose. Because the 0.25 mg/m sq dose of IFN-gamma has been studied in a phase II study in patients with metastatic malignant melanoma and found to have a low response rate, we did not further pursue investigation of the effectiveness of 0.1 mg/m sq of IFN-gamma in patients with metastatic melanoma.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI

OBJECTIVES

1. To explore the immunological effects and toxicity of different doses of recombinant interferon (IFN)-gamma in patients with metastatic malignant melanoma.

2. To record the antitumor effects of these IFN-gamma regimens in patients with metastatic malignant melanoma.

3. To estimate the response rate to the most effective immunomodulatory regimen of recombinant IFN-gamma determined from the first part of this study in patients with metastatic malignant melanoma.

METHODS EMPLOYED

Patients who met the following criteria were 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 clinical evaluation, patients were assigned to one of three groups. Each group received a different dose of IFN-gamma with the first group receiving 0.01 mg/m^2, the second 0.1 mg/m^2, and the third 0.25 $mg/^2$. Patients received IFN-gamma by daily subcutaneous injection for a period of 2 months. During the course of treatment, frequent blood sampling was performed to assess the effects of treatment on the immune system.

Eighteen patients were entered onto the study, ten males and eight females. The median age was 51 years with a range of 27 to 75 years. Five patients were treated at each dose level mentioned previously. At each dose level, three patients had predominantly non-visceral disease and two patients had predominantly visceral disease.

MAJOR FINDINGS

The IFN-gamma treatment was fairly well-tolerated with the exception of the following patients experiencing significant toxicity. One patient at the 0.1 mg/m^2 dose level experienced worsening of subclinical dermatomyositis and was removed from study. Three patients at the 0.25 mg/m^2 dose level had a Grade III decrease in granulocytes (less than 1,000 $cells/mm^3$) and required a dose reduction. One patient had Grade IV elevation in his hepatic enzymes and was taken off study. One patient refused to continue treatment and stopped therapy after only a few doses. Chills, fever, headache, nausea, vomiting, diarrhea, fatigue, myalgia, arthralgia, and paresthesias were seen frequently but were all Grade I or II.

Response to treatment was as follows: Three patients had significant progression of their disease after 1 month of treatment and were removed from study. Seven patients had evidence of progressive disease after 2 months of treatment and stopped therapy at that point. Two patients had stable disease after 2 months of treatment and continued treatment for 4 and 5 months, respectively at which time they showed evidence for progressive disease. One patient who had stable disease for 6 months was removed from study because he developed new congestive heart failure that was not related to the IFN-gamma treatment. One patient treated at the 0.25 mg/m^2 had progressive disease at 1 month of treatment; however, when he returned 1 month later he had a response to treatment and so was placed back on the study and continued on treatment. His metastatic lesions continue to shrink and now qualify him for a partial response. A decision was made recently to stop treatment because he was found to have a new atrial mass. At cardiac surgery, this mass turned out to be metastatic melanoma.

Immunological assays designed to assess monocyte activation were performed in each group of five patients at the three different dose levels. Monocyte activation was consistently observed at the two higher dose levels of 0.1 mg/m^2 and 0.25 mg/m^2. Statistical analysis showed no significant differences between these two dose levels in terms of monocyte activation.

SIGNIFICANCE

A prior study at the Biological Response Modifiers Program of IFN-gamma in patients with low tumor burdens (that is, rendered disease free by surgery but at high risk for recurrence) demonstrated that a dose of IFN-gamma substantially lower than the maximum tolerated dose brought about equal or greater immunomodulatory effects than the maximum tolerated dose. We tested three different doses in this study of patients with metastatic malignant melanoma and relatively large tumor burdens in order to determine if the same phenomena existed in this group of patients. This study shows that in spite of their significant tumor burdens, patients with metastatic melanoma can have marked increases in the degree of monocyte activation. However, unlike the previous study, there appeared to be no superiority of the 0.1 mg/m^2 dose over the 0.25 mg/m^2 dose in terms of monocyte activation.

PROPOSED COURSE

Because there was no difference the 0.1 mg/m^2 dose and the 0.25 mg/m^2 dose, the study was terminated because a phase II study of IFN-gamma in malignant melanoma patients with metastatic disease has been performed at the 0.25 mg/m^2 dose and showed a response rate of only 11%.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09331-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. E. Janik	Expert	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*

Frederick Memorial Hospital (C. Donovan); Program Resources, Inc., Frederick, MD (W. J. Urba, J. Beveridge); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 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 biologic 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 E. Janik	Expert	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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 (creatinine < 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-five patients were entered on the trial. Twenty-four completed treatment; 1 was taken off study early due to toxicity (grade 3 fatigue

attributed to IL-2). Three others were taken off study at later intervals due to toxicity (fatigue, exacerbation of arthritis). No partial or complete responses were seen; one patient with renal cancer evidenced a minor response. Analysis of immune modulatory effects indicates that poly ICLC had no effect on circulating NK and LAK cell activity and, when given in combination with IL-2, did not increase NK or LAK cell activity above the levels which would have been expected with IL-2 alone.

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

This trial has been closed to further entry. Further trials with poly ICLC will explore its combination with other cytokines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09332-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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. G. Steis	Medical Officer	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	W. H. Sharfman	Expert	CRB, NCI
	J. J. Oppenheim	Chief	LMI, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol); Frederick Memorial Hospital, Frederick, MD (N. Wells)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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) plays a central role in immune responses and might be useful in the treatment of cancer patients for several reasons. We conducted a phase I clinical trial of IL-1 alpha to determine the: (1) toxicity of IL-1 in cancer patients, (2) effects of IL-1 in the immune system, (3) effects of IL-1 on the hemopoietic system, (4) pharmacokinetics of IL-1 and (5) the effect of adding indomethacin on all of the above.

All patients had chills, fever, headache, and some had nausea, vomiting, myalgia, arthralgia, and abdominal pain. Hypotension, renal insufficiency, confusion, and abdominal pain were the dose-limiting toxicities. The inpatient-maximum tolerated dose (MTD) of IL-1 alpha administered alone with blood pressure support was determined to be 0.3 mcg/kg. With the addition of indomethacin at 25 mg every 8 hours, the maximum tolerated dose was determined to be 0.1 mcg/kg. IL-1 alpha did not cause an increase in peripheral blood natural killer cell and lymphokine activated killer cell activity. However, IL-1 treatment did induce increases in serum soluble IL-2 receptor levels and in IL-6 levels. Pharmacokinetic studies indicated measurable IL-1 alpha levels at the two highest dose levels consistent with a short alpha half-life of 10-15 minutes. IL-1 caused a dose related 2-7 fold increase in the white blood count (mainly increased neutrophils and neutrophil bands). The platelet count declined slightly during treatment but increased one and a half to two times above baseline one week later in five patients studied at that time. Bone marrow aspirates one day after the end of treatment showed increased cellularity and increased M/E ratio compared to ones obtained before treatment.

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, BRMP, NCI
William H. Sharfman	Expert	CRB, NCI
Joost J. Oppenheim	Chief	LMI, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI

OBJECTIVES

1. To determine the toxicity of and the maximally tolerated dose (MTD) of IL-1 (interleukin-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 were eligible for this trial if they had a histologically confirmed solid tumor that was 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. Patients were required to 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 their initial clinical evaluation, patients were admitted to the hospital and treated with IL-1 alpha given by intravenous infusion over 15 minutes daily once a day for 7 consecutive days. Cohorts of three patients were treated with IL-1 alpha at escalating dose levels until intolerable (dose limiting) toxicity was observed in two patients. The dose below that was defined as the MTD. At that point, subsequent patients were treated with indomethacin 25 milligrams every 8 hours plus IL-1 alpha beginning with a dose level 2 doses below the previously established MTD. Three patients each were treated with IL-1 alpha alone at the following dose levels: 0.01, 0.03, 0.1, 0.3, and 1.0 mcg/kg.

In the group of patients who received indomethacin plus IL-1 alpha, three patients were treated at the 0.03 mcg/kg dose level, 3 patients at 0.1 mcg/kg, six patients at 0.3 mcg/kg and one patient at 1.0 mcg/kg. The 28 patients had a median age of 49 years with a range of 26 to 68 years. Eighteen patients had received prior chemotherapy, one prior immunotherapy, and nine no prior treatment. Thirteen patients had a gastrointestinal malignancy, three each had breast cancer or melanoma, two patients each had lung cancer, ovarian cancer or head and neck cancer, and one patient each had mesothelioma, sarcoma or prostate cancer.

MAJOR FINDINGS

IL-1 alpha caused fever, chills, headache, and fatigue in most patients. Myalgia, arthralgia, nausea, vomiting, diarrhea, and abdominal pain also occurred, but less frequently. Phlebitis of peripheral veins was noted in all patients at the second dose level so subsequent treatments were administered via a central venous catheter. These side effects were mostly Grade II or less except for fever which was occasionally Grade III (greater than 40^{o}C). A few patients experienced photophobia, mucositis, transient dyspnea or crampy abdominal pain. Hypotension was noted with all but the lowest dose level and was more severe with the higher doses. Beginning with the 0.3 mcg/kg dose level, most patients required intravenous pressors (phenylephrine) in addition to intravenous fluids to support their blood pressure. In several patients treated at the higher dose levels, renal insufficiency manifested by a rising creatinine was noted. In several instances, the creatinine value returned to normal in spite of continued treatment. In all cases, the creatinine returned to baseline after therapy was stopped. Serial determinations of fractional sodium excretion indicated a prerenal etiology for this transient renal insufficiency.

Dose-limiting toxicity was noted at 1.0 mcg/kg of IL-1 alpha alone and consisted of Grade IV hypotension in one patient and Grade III renal insufficiency in another. Therefore, the inpatient MTD of IL-1 alpha alone was 0.3 mcg/kg. In the second group of patients treated with indomethacin plus IL-1 alpha, one patient sustained a myocardial infarction at the 0.3 mcg/kg dose level and another patient at the same dose level had severe abdominal pain with rebound tenderness as well as Grade III neurotoxicity (confusion). One patient had Grade III neurotoxicity consisting of agitation and somnolence at the 1.0 mcg/kg dose level. The MTD of IL-1 alpha plus indomethacin was determined to be 0.1 mcg/kg. Indomethacin decreased the peak temperature and the severity of myalgia and arthralgia in some patients but did not change the severity of the hypotension. There were no bleeding complications and no greater degree of renal toxicity in patients treated with indomethacin.

In addition to the rises in serum creatinine, patients were also noted to have increases in liver function tests with the higher doses of IL-1 alpha. The total protein and albumin were decreased and the serum potassium was also decreased in several patients. No patients experienced an increase in the serum calcium or a decrease in serum glucose. Antitumor activity was noted in one patient initially thought to have adenocarcinoma of unknown primary metastatic to the lungs. Subsequent work-up revealed that the patient had metastatic prostate cancer. The transient decrease in the patient's testosterone level

after IL-1 treatment was probably the mechanism of antitumor activity. The patient subsequently was treated with standard hormonal manipulation for metastatic prostate cancer.

IL-1 alpha caused striking dose related hematologic effects. The peripheral white blood count (wbc) increased 2-7 times above baseline. Increases were noted at the four hour post-treatment time point and persisted at the 24 hour time point. The wbc increase tended to decline over the last three days of therapy. The wbc increase consisted mainly of neutrophils and neutrophilic bands. The platelet count declined slightly during treatment but was noted to be one and a half to two times above baseline one week later in five patients treated with IL-1 alpha plus indomethacin. Bone marrow aspirates obtained before treatment and one day after seven days of treatment with IL-1 alpha showed a marked increase in cellularity and an increase in the M/E ratio. The frequency of colony-forming unit cells was decreased after treatment compared to baseline in most patients.

IL-1 alpha treatment did not induce any detectable colony stimulating factors or interferon gamma. Tumor necrosis factor was present in four patients before treatment and increased slightly with treatment in one patient, while three patients had slight decreases during therapy. Serum soluble IL-2 receptor levels increased with treatment in a dose-related fashion two to five times above baseline starting with the 0.03 mcg/kg dose level. IL-2 was detectable in the serum of three patients before treatment and decreased slightly with treatment in two patients whereas it increased slightly during treatment in one patient. IL-6 was detectable in the serum of patients after treatment starting at the 0.03 mcg/kg dose level. It was present beginning 2 hours after treatment and peaked 2-4 hours after the IL-1 alpha dose. IL-6 levels were higher with higher doses of IL-1 alpha. Measurable IL-1 levels were observed at the two highest dose levels. Pharmacokinetic data indicated a short alpha half-life of 10-15 minutes. Natural killer and lymphokine activated killer assays showed transient decreases at the four-hour post-treatment time point concurrent with a marked decrease in the number of circulating LEU-19+ cells.

SIGNIFICANCE

This phase I study of IL-1 alpha demonstrates that this agent is toxic to humans at very low doses. Hypotension, renal insufficiency, confusion, and abdominal pain were the dose-limiting toxicities. The inpatient-MTD of IL-1 alpha administered alone with blood pressure support is 0.3 mcg/kg. With the addition of indomethacin at 25 mg every 8 hours, the MTD is 0.1 mcg/kg.
IL-1 alpha did not cause an increase in peripheral blood natural killer cell and lymphokine-activated killer cell activity. However, IL-1 treatment did induce increases in serum soluble IL-2 receptor levels and in IL-6 levels. IL-1 alpha causes significant hematological effects consisting of a marked increase in peripheral wbc and an increased cellularity in the bone marrow. Data from five patients studied one week after treatment ended indicated IL-1 induced an increase in the platelet count.

PROPOSED COURSE

This phase I study is now complete. Because of preclinical data suggesting that a twice-a-day schedule of IL-1 alpha may be more beneficial in terms of accelerating wbc and platelet count recovery after chemotherapy, we intend to amend the protocol to study a twice-a-day administration schedule of IL-1 alpha. Two follow-up protocols have also been written that will allow us to investigate the antitumor efficacy of IL-1 alpha in patients with metastatic malignant melanoma as well as to determine the role of IL-1 alpha in preventing or reducing bone marrow suppression from antineoplastic chemotherapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09334-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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
	J. E. Janik	Expert	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

OAD, BTS, BRMP, NCI, Bethesda, MD (J. Ashwell); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol); Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (T. Watson)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 undertook a Phase I study of anti-CD3 monoclonal antibodies in patients with solid tumors to determine the toxicity and immunomodulatory properties of this antibody at various doses and schedules. Initially, the protocol was designed to treat five patients at one of six dose levels including 1, 10, 30, 100, 300 and 1,000 micrograms (mcg) per patient. Patients received four doses by three-hour infusion over two weeks. Dose-limiting toxicity occurred in all three patients at 100 mcg of anti-CD3 and further dose escalation was discontinued. Dose-limiting toxicity consisted of severe headache associated with a syndrome of aseptic meningitis. Cerebrospinal fluid showed an abnormal lymphocytosis with an elevated protein. Thus, the maximum tolerated dose of anti-CD3 by three-hour infusion was 30 mcg. An additional five patients received 30 mcg by bolus injection according to the same schedule and severe headache was again a serious problem. A preliminary review of the immunologic data failed to reveal marked changes according to this schedule. Because of the toxicity at lack of immunologic effects when anti-CD3 is administered according to this schedule, we changed to daily administration for 14 days. We treated five patients with three mcg of anti-CD3 by bolus and two by three-hour infusion. Severe headache was seen in both patients receiving 3 mcg by three-hour infusion and in 1/5 patients receiving bolus administration. Toxicity appears to be related to the dose, duration of infusion and frequency of anti-CD3 administration.

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
John E. Janik	Expert	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
William H. Sharfman	Expert	CRB, 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.

In the first phase of the study, 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 (mcg) per patient. Patients received four doses of antibody given three days apart.

In the second phase of the study, five patients received 30 mcg of anti-CD3 by intravenous (i.v.) bolus according to the same schedule. In the third phase of the study, an additional five patients received 3 mcg by i.v. bolus daily for 14 days and two patients received 3 mcg by three-hour infusion.

The majority of monoclonal antibody infusions were administered in the outpatient clinic. 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 biopsies performed to examine effects of treatment on tumor histology.

MAJOR FINDINGS

Five patients were treated at the 1 mcg dose level and five patients were treated at the 10 mcg dose level; all ten patients received all four doses of treatment. Five patients received 30 mcg 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 mcg dose, one patient received two doses and two patients received one dose each. Since dose-limiting toxicity was observed at 100 mcg of anti-CD3, an additional five patients were treated at the 30 mcg dose level for a total of 10 patients at the 30 mcg dose level. The toxicities observed were dose-related. Patients receiving one mcg of antibody experienced no significant toxicities. Patients receiving 10 mcg 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 mcg dose was associated with mild headaches in most patients easily controlled with acetaminophen but severe headache prompted the performance of a spinal tap in two patients. These patients had an abnormal number of lymphocytes in the spinal fluid and an elevated protein. All cultures for bacteria and viruses were negative and the patient's symptoms resolved spontaneously. At 100 mcg, 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. No bacterial or fungal organisms were cultured from the spinal fluid.

Because of the toxicity observed administering anti-CD3 by three-hour infusion, we elected to treat five patients at our MTD (30 mcg) by bolus infusion. Among these five patients, two patients developed severe headache requiring discontinuation of treatment.

A preliminary analysis of the immunologic effects failed to reveal significant changes in the cell surface phenotype of peripheral blood mononuclear cells at doses below 100 mcg. There were no changes in T-cell number or phenotype. There were no increases in IL-2 receptor expression. At the 100 mcg dose level, there was marked lymphopenia during therapy that resolved with discontinuation of therapy. At 10, 30 and 100 mcg doses, there were transient increases observed in the peripheral granulocyte counts. Anti-CD3 did not cause significant changes in the ability of peripheral blood mononuclear cells to proliferate in response to Con A, IL-2, anti-CD3 or alloantigens. Minor increases in serum IL-2 receptor levels were observed in an occasional patient. Induction of interferon-gamma, tumor necrosis factor or granulocyte-colony stimulating factor was not observed.

Since toxicity was prominent and immunomodulatory effects were minor, we elected to examine the effects of more frequent administration of anti-CD3 at a variety of doses. Patients were to be treated with anti-CD3 daily for 14 days by i.v. bolus or three-hour infusion. Two patients received 3 mcg of anti-CD3 by three-hour infusion and neither patient could complete all 14 days because of headache. Five patients received 3 mcg of anti-CD3 by i.v. bolus and two patients had therapy prematurely discontinued because of headaches. More

frequent administration of anti-CD3 seemed to further decrease the dose of antibody that could be safely tolerated. The immunomodulatory effects of these doses of anti-CD3 have not been determined.

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. Increasing the frequency of anti-CD3 appears to lower the maximally tolerated dose.

PROPOSED COURSE

This study has been closed to further accrual. Analysis of the immunological changes is being performed and further studies will be conducted based on their outcome.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09335-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	S. P. Creekmore	Chief	BRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	K. C. 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); OAD, BTS, BRMP, NCI, Bethesda, MD (J. Ashwell)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 three patients have been treated on this protocol and each patient received 1 microgram of anti-CD3. Two patients did not have any toxicity, whereas one patient had treatment discontinued after four doses because of a severe bifrontal headache. None of the patients had a tumor response during monoclonal antibody treatment. There were no significant immunological changes noted in the patients' peripheral blood during treatment.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Stephen P. Creekmore	Chief	BRB, 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

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 three patients have been treated on this protocol so far and each patient was 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 three patients were entered on this study and all are evaluable for toxicity and response. All three patients had mycosis fungoides. Two patients received all ten doses of therapy and one patient received only four doses before treatment was stopped because of severe headaches. All patients experienced 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 09336-02 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, end institute affiliation)*

PI:	D. L. Longo	Associate Director	OAD, BRMP, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, BRMP, 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-FCRDC, 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 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 eight 2-week cycles, for a total of sixteen weeks. To date, there have been 23 evaluable patients entered on study: nineteen have achieved a complete response (83%). There have been 6 relapses (32%), all 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, BRMP, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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, 16-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 eight two-week cycles for a minimum total duration of 16 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 5, the patient is completely restaged. All tests that were positive initially are repeated. If the patient is in complete remission, 3 more cycles of therapy are given. Otherwise, patients are treated for three 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-eight patients have been entered on this study. Five are too early to evaluate. Of the 23 evaluable patients, 19 (83%) have achieved a complete response. Six patients (32%) have relapsed from complete remission, all after short remissions (less than 10 months). Two relapsed patients have died: one patient underwent autologous bone marrow transplant, relapsed and then received allogeneic bone marrow transplant, but died immediately post transplant due to pneumonia and overwhelming sepsis, free of lymphoma. One other patient died of progressive disease following first relapse. Among the other four relapsed patients, two have undergone autologous bone marrow transplant and are in second CR; one other has received involved field radiation and is in clinical CR and one patient has undergone standard combination chemotherapy and is alive with disease. Four patients have died on this study (17%): two of the four induction failures and 2 relapsed patients. Of the two living patients who never achieved a CR, one is alive with only indolent lymphoma, a microscopic finding at restaging laparotomy, and one patient is currently undergoing radiation therapy for progressive disease in the mediastinum. 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. We have demonstrated the ability to give a more dose-intense ProMACE-CytaBOM with no additional toxicity.

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 09337-02 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*

Radiation Oncology Branch, NCI, Bethesda, MD (E. Glatstein); Laboratory of Pathology, NCI, Bethesda, MD (E. S. Jaffe); Program Resources, Inc., Frederick, MD; Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 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, IVA (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 ameliorate the expected severe myelotoxic effects of these increased drug doses. To date, 5 of the 6 evaluable patients have achieved a complete response. The patient who was an induction failure has since undergone an allogeneic bone marrow transplant and achieved a complete response. All evaluable patients are alive and free of disease at this time. No unexpected toxicities have been observed with this treatment.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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

To date, eight patients have been enrolled in the study and 6 are evaluable. (One patient is too early to evaluate and one patient dropped out in cycle 2 for reasons unrelated to treatment.) Of the 6 evaluable patients, 5 have achieved a complete response. The patient who was an induction failure has since undergone an allogeneic bone marrow transplant and achieved a complete response. All evaluable patients are alive and free of disease at this time. No unexpected toxicities have been observed. Despite the GM-CSF, some dose reductions have been necessary.

SIGNIFICANCE

DIMOPP with GM-CSF can be safely administered to patients with Hodgkin's disease. Some dose reductions are necessary due to myelosuppression and/or thrombocytopenia. The toxicity of GM-CSF (flu-like symptoms) although expected, is significant in some patients and has required dose-reduction in several. The actual dose-intensity delivered with DIMOPP is greater than the actual dose-intensity with standard MOPP.

PROPOSED COURSE

The study remains open to patient 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 09339-02 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	P. L. Duffey	Research Specialist	OAD, BRMP, NCI
	BRMP Staff		BRMP, NCI

COOPERATING UNITS *(if any)*
Radiation Oncology Branch, NCI, Bethesda, MD (E. Glatstein); Laboratory of Pathology, NCI, Bethesda, MD (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, 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 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 54 evaluable patients randomized to MOPP, 52 (96%) achieved a complete response; 7 relapsed. Forty-nine of fifty-one randomized radiation-treated patients (96%) achieved a complete response: 17 patients (35%) relapsed. Four MOPP-treated patients (7%) and ten (20%) radiation-treated patients have died.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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. During the early years of the study, patients with III_A, and massive mediastinal disease were randomized, but were excluded from further randomization after interim analysis showed an unacceptably high failure rate for these patients treated with radiation. 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 iv days 1,8, vincristine 1.4mg/M2 iv 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. Seventeen patients (35%) have relapsed from CR. Among the 17 patients relapsing from a radiation therapy-induced CR, 1 received MOPP-ABVD chemotherapy and failed to obtain a second complete response. Sixteen patients were treated with MOPP; 11 (73%) achieved a complete remission and 8 (53%)

continue in second remission for periods ranging from 2-11 years. One patient died in second remission. Two patients relapsed from second remission and one is in a third remission after high dose therapy and bone marrow transplantation. There have been a total of 10 deaths (20%): 7 with progressive disease and 3 with no evidence of disease (one sepsis during MOPP reinduction, 1 AIDS, 1 pneumonia).

Randomized to MOPP:

Fifty-five patients have been randomized to MOPP. One patient died from a myocardial infarction during cycle 1 with normal blood counts. His death is included in the survival curve: however, he is considered inevaluable for response. Fifty-two of 54 patients (96%) achieved a complete remission with MOPP. Seven (13%) patients have relapsed. Of the 7 patients who have relapsed from a MOPP-induced CR, 5 were reinduced with radiation. All 5 achieved a second CR, but only 2 of the 5 remain in second remission; 3 have relapsed. Two patients received a second course of MOPP, one of whom achieved a second CR (5+ years), and one of whom died during second remission induction. Four patients (7%) have died, 3 with Hodgkin's disease (including the patient dying during cycle 1) and 1 died free of disease.

Stage IA's-nonrandomized to Radiation:

Thirty patients with peripheral stage IA Hodgkin's disease were treated nonrandomly with radiation therapy. All 30 achieved a complete response and none has relapsed. Two patients have died free of disease (one from myocardial infarction and one from suicide) and one is lost to follow-up.

SIGNIFICANCE

For the two randomized arms of the study, the complete response rates are equivalent. However there are significantly fewer relapses with the MOPP treatment. The projected 10-year disease-free survival for radiation therapy-treated patients is 60% and for MOPP-treated patients is 86% (P2=0.009 in favor of MOPP). The projected 10-year overall survival for patients randomized to radiation therapy is 76% and for MOPP-treated patients is 92% (P2=0.051) in favor of MOPP. When patients with III_A, and massive mediastinal disease are excluded from analysis, the advantage for MOPP is no longer statistically significant. 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09340-02 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	P. L. Duffey	Research Specialist	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*
Radiation Oncology Branch, NCI, Bethesda, MD (E. Glatstein), Laboratory of Pathology, NCI, Bethesda, MD (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) Minors

☐ (a2) Interviews

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 29% 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.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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 142 patients entered on study with 125 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 60 evaluable patients on this arm, 45 achieved a CR (75%). There have been 14 relapses (31%) and 16 (27%) have died. Twenty-nine patients (48%) 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 58 evaluable patients on this arm. Twenty-seven patients remain on WW and are alive with lymphoma. Thirty-one patients (53%) have crossed-over to intensive therapy. 9/31 (29%) patients have achieved a complete response and 4 (44%) have relapsed from CR. Fourteen patients (24%) 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: Twenty-three patients who were not suitable for randomization were treated with intensive therapy at diagnosis. Four continue on therapy and are too early to evaluate. Of the 19 patients evaluable for response, 11 (58%) have achieved a complete response. Four of 11 (36%) have relapsed and 8 of the 19 (42%) have died.

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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09341-02 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*
Institute of Allergy and Infectious Diseases, Bethesda, MD (A. S. Fauci); Laboratory of Pathology, NCI, Bethesda, MD (E. S. Jaffe); Program Resources, Inc., Frederick, MD; Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, 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. All four patients have achieved a complete response and 1 has relapsed. The relapsed patient has since undergone bone marrow transplantation and remains in second CR. No patient has died. More patients and longer follow-up are necessary to draw any conclusions.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
BRMP Staff		BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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 Pneumocystis 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. All four patients have achieved a complete response and 1 has relapsed. The relapsed patient has since undergone bone marrow transplantation and remains in second CR. No patient has died. 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 09342-02 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	P. L. Duffey	Research Specialist	OAD, BRMP, NCI
	BRMP Staff		BRMP, NCI

COOPERATING UNITS *(if any)*
Radiation Oncology Branch, NCI, Bethesda, MD (E. Glatstein), Laboratory of Pathology, NCI, Bethesda, MD (E. S. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, 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 55 evaluable patients entered on study of whom 53 (96%) achieved a complete remission. There has been one relapse. 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.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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 I 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. Fifty-three of fifty-five (96%) patients achieved a complete response and one has 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. Approximately forty percent of patients had no dose reductions and 40% had only one to three dose reductions. Side effects with this therapy are minimal. There were only 11 hospital admissions out of 230 courses of therapy given.

SIGNIFICANCE

With a complete response rate of 96% and a relapse rate of 2% 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 modified 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09343-02 CRB

PERIOD COVERED: October 1, 1989 through September 30, 1990

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, BRMP, NCI
Others:	P. L. Duffey	Research Specialist	OAD, BRMP, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	W. Wilson	Special Assistant	OD, NCI

COOPERATING UNITS *(if any)*

Radiation Oncology Branch, NCI, Bethesda, MD (E. Glatstein); Laboratory of Pathology, NCI, Bethesda, MD (E. Jaffe)

LAB/BRANCH: Clinical Research Branch

SECTION:

INSTITUTE AND LOCATION: NCI-FCRDC, 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.)*

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 five years. There have been 10 relapses. A total of 14 patients have died; eight had no evidence of lymphoma although six were treatment-related deaths. Overall, of the 23 patients, seven 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, BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, 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 and cisplatin.

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 and cisplatin 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 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 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; leucovorin 50mg/M^2 iv beginning 24 hours after the initiation of the MTX infusion, followed by oral leucovorin 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 and cisplatin: VP16 100mg/M^2 iv daily for 5 days; cisplatin 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/cisplatin, achieved a CR, but died from sepsis. Of the twenty-one CR's, ten have relapsed. There have been fourteen deaths: six patients with lymphoma, eight patients died with no evidence of lymphoma. Overall, seven patients are alive and continue in complete response.

SIGNIFICANCE

This study demonstrates that about one third 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.

PUBLICATION

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 09350-01 CRB

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
A Phase Ib Trial of Levamisole Alone and In Combination with rIFN-Gamma

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. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller); Frederick Memorial Hospital, Frederick, MD (J. Hursey); Genentech, Inc., San Francisco, CA (H. Jaffe)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, 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.)*

The purpose of this protocol was to take a second look at Levamisole using modern immunological assays in order to determine if there would be a dose of Levamisole or a schedule of Levamisole administration that would produce maximal immunomodulation. This trial was also designed to combine Levamisole with interferon gamma, a cytokine whose immunostimulating properties has been well defined in two previous trials conducted at the Biological Response Modifiers Program.

This trial investigated four different doses of Levamisole administered once a day every other day. Two groups of patients were treated, one with small tumor burdens in the adjuvant setting and another group with large tumor burdens in the advanced disease setting. After two weeks of treatment with Levamisole alone, all patients then were treated with the same dose of Levamisole plus interferon gamma 0.1 mg/m sq subcutaneously every other day.

This study determined that Levamisole was toxic at the 10 mg/kg dose level in both groups of patients. A maximum tolerated dose therefore was defined to be 5 mg/kg every other day. At this dose level, treatment was well tolerated with Levamisole alone and with Levamisole plus interferon gamma. Analysis of the immunological assays that have been performed to date indicate that Levamisole enhances natural killer cell activity in a dose-dependent manner. There was a suggestion that the activity was better in the adjuvant setting. Soluble interleukin-2 receptor levels were increased during the combined therapy.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI

OBJECTIVES

1. To determine the maximum tolerated dose (MTD) of Levamisole alone and in combination with interferon (IFN) gamma.

2. To determine the optimal immunomodulating dose of Levamisole alone and in combination with IFN-gamma.

3. To compare effects of Levamisole plus IFN-gamma to IFN-gamma alone (historical data).

4. To study all of the above in the adjuvant setting and in patients with advanced cancer.

5. To note any antitumor effects.

METHODS EMPLOYED

Patients were eligible for this trial if they had histologically confirmed solid tumor, Hodgkin's disease, or non-Hodgkin's lymphoma that had failed to respond to standard treatment regimens. Patients in the adjuvant setting needed to start treatment within 6-8 weeks of their last surgery. Patients with melanoma were eligible if they were Stage IIA, Stage IIB, or Stage III or had a recurrence of their disease that had been surgically resected and had no evidence of disease elsewhere. Renal cell cancer patients were eligible if they had completely resected Stage II or Stage III disease. All patients were required to have a Karnofsky performance status of equal to or greater than 70%, adequate organ function, no history of myocardial infarction, coronary artery disease, congestive heart failure, or ventricular arrhythmia requiring treatment, no history of central nervous system metastases, and no treatment four weeks prior to entering the study. Patients with advanced cancer were required to have evaluable or measurable disease.

Initial clinical evaluation included a complete history and physical examination, serum blood chemistries and complete blood count, coagulation studies, urinalysis, electrocardiogram, chest x-ray, CT of the brain, and baseline immune parameters times three. In patients with metastatic disease, imaging studies were performed as clinically indicated.

Patients were treated according to the following plan. All patients received Levamisole alone once a day every other day for a two-week period (a total of six doses). The patients then had ten days of rest after which they resumed treatment but this time with IFN-gamma 0.1 mg/m^2 subcutaneously every other day along with the same dose of Levamisole that they had received before. Patients with advanced cancer were treated with the combination of the two agents for a total of one month. Patients in the adjuvant setting were treated with a combination of the two agents for a total of three months. Patients were entered in cohorts of five at escalating dose levels until the MTD was defined. The doses of Levamisole that were used were 1.0, 2.5, 5.0, and 10.0 mg/kg.

In addition to serial complete blood counts and serum chemistry studies, all patients had frequent immune parameters obtained. Assays that were performed included natural killer (NK) cell activity, lymphokine activated killer cell activity, serum soluble interleukin-2 receptor level, serum IFN-gamma level, serum neopterin level, monocyte hydrogen peroxide production, and cell surface expression of Fc receptor and class II molecules.

MAJOR FINDINGS

This protocol enrolled 20 patients in the advanced setting and 20 patients in the adjuvant setting. One patient in the advanced setting is not evaluable because of the early removal secondary to rapid disease progression. There were 10 males and 10 females in the advanced setting, 14 males and 6 females in the adjuvant setting. The median age was 59 years with a range of 36 to 75 in the adjuvant setting, the median age was 43 years with a range of 19 to 75 in the adjuvant setting. In the advanced setting, 11 patients had malignant melanoma, 2 renal cell carcinoma, 4 colon cancer, 2 breast cancer, and 1 patient had malignant chordoma. In the adjuvant setting, 17 patients had melanoma and 3 patients had renal cell carcinoma.

Dose-limiting toxicity was observed in three of five patients treated in the adjuvant setting at 10 mg/kg of Levamisole. Toxicity included Grade III fever, chills, nausea, vomiting, dehydration, headache, rash, and fatigue. Dose-limiting toxicity was also observed at 10 mg/kg in patients with advanced disease. One patient had Grade III hypertension and one patient had Grade IV vomiting, Grade III fever, fatigue and rash. At the three lower dose levels, toxicity from Levamisole alone was mild and consisted mainly of Grades I and II side effects such as metallic taste, nausea, vomiting, rash, dizziness, headache, fatigue, fever, arthralgia, myalgia, stomatitis, constipation, chills, and mild central nervous system side effects. Patients treated with Levamisole plus IFN-gamma had more fever and chills, myalgia, arthralgia, and headache. In addition, a few patients experienced paresthias, nausea, vomiting, diarrhea, constipation, and mild central nervous system side effects such as insomnia or anxiety. One patient had Grade III granulocytopenia during treatment of IFN-gamma. The toxicities were similar in the adjuvant and advanced disease groups. The MTD of IFN-gamma plus Levamisole was determined to be 5 mg/kg of Levamisole plus IFN-gamma 0.1 mg/m^2. At the MTD of Levamisole, all toxicities were Grade I or II except for Grade III fatigue and Grade III headache in one patient.

All of the immunological assays have not been run on these patients but data obtained to date indicates the following. Levamisole enhanced NK cell activity in a dose-dependent manner. There was a suggestion that activity was best in the adjuvant setting. The increase in NK activity was not affected by the addition of IFN-gamma. No increases in LAK cell activity were observed. Levamisole did not induce serum IFN-gamma levels. Serum-soluble interleukin-2 receptor levels did increase during combined therapy.

SIGNIFICANCE

Although Levamisole has been investigated in cancer patients for many years, only recently have several positive trials been reported that demonstrated a benefit of Levamisole in patients treated in the adjuvant setting who had colon cancer or malignant melanoma. Investigation to date indicates that Levamisole is probably not working directly but instead indirectly, probably via the immune system. Most immunological studies of Levamisole were performed in the 1970's and used less sophisticated assays than are available today. The dose and schedule of administration for the previous clinical trials were arbitrarily chosen. We surmised that a different dose and/or schedule might produce better immunomodulatory effects. In addition, we speculated that Levamisole might have additive or synergistic immunomodulating effects with other cytokines.

This protocol was performed in order to take a second look at Levamisole's immunomodulating properties and define them better using modern assays. We wanted to examine a range of Levamisole doses and a schedule using Levamisole every other day. We wanted to compare the activity in patients with small and large tumor burdens and finally we wanted to combine Levamisole with IFN-gamma, an agent whose immunomodulating properties were well defined in both groups of patients.

This trial demonstrates that Levamisole can be given in higher doses and more frequently than has been customarily done in previous trials with Levamisole. In contrast to previous reports suggesting that Levamisole was only an immunorestorative agent, this trial demonstrates that Levamisole can boost NK activity in patients with low tumor burdens and relatively intact immune systems. In fact, preliminary data obtained so far suggest that enhancement of NK activity was better in patients treated in the adjuvant setting.

All of the immunological assays have not been completed. It is possible that once all the data is obtained, we will be able to define an optimal immunomodulatory dose of Levamisole that is significantly different from that used in clinical trials to date. If that is the case, it is possible that using Levamisole at this different dose and schedule might produce even better antitumor effects than those that have been observed to date.

PROPOSED COURSE

This clinical trial is currently continuing to enroll patients in the adjuvant setting to define changes in immunological assays three and six hours after treatment. Five patients each will be enrolled at the 1.0, 2.5 and 5.0 mg/kg

dose levels. After accrual to the study has been completed, the immunological assays will be analyzed. It is likely that we will amend the protocol to treatment of patients with a combination of Levamisole plus interleukin-2 in order to determine the effects on the immune system of that combination.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09351-01 CRB

PERIOD COVERED: October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Phase IB Study of R24 MoAb Given in Conjunction with IL-2 and LAK Cells

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

PI:	M. Sznol	Principal Investigator	CTEP, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	J. E. Janik	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*: Program Resources, Inc., Frederick, MD (W. J. Urba)

LAB/BRANCH: Clinical Research Branch

SECTION:

INSTITUTE AND LOCATION: NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 1.5	OTHER: 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 response rate in patients with metastatic melanoma receiving interleukin-2/lymphokine activated killer cells (IL-2/LAK) is approximately 20%. Tumor-specific monoclonal antibodies can potentiate the antitumor activity of IL-2/LAK in preclinical animal models. Therefore we are initiating a trial adding R24, a murine monoclonal antibody reactive with melanoma tumor cells expressing the ganglioside GD3, to a modified IL-2/LAK regimen. Escalating doses of R24 are administered by 24-hour continuous infusion to cohorts of patients on day 0. The R24 is followed by IL-2 administered as a continuous infusion on days 1-6, followed by a second infusion on days 11-18 given concurrently with LAK cells on days 11, 12 and 14. All patients entering the study have tumor biopsied before treatment, prior to the beginning of IAK infusions, and following the completion of IL-2/LAK. Tumor tissue is tested for R24 binding and antigen saturation, immunophenotyping of tumor infiltrating lymphocytes, expression of tumor-associated antigens and MHC class 1 and 2 molecules, and if possible, expansion and functional characterization of tumor infiltrating lymphocytes. Assays of peripheral blood lymphocytes include phenotyping, measurement of cytotoxicity against melanoma cell lines, and quantitation of proliferation when stimulated with R24. Six patients have been accrued at the first dose level of R24 (3 mg/m2). Accrual will continue until a maximum tolerated dose of R24 in combination with IL-2/LAK is reached.

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Principal Investigator	CTEP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI

OBJECTIVES

To determine the maximum tolerated dose of R24 when combined with a continuous infusion regimen of interleukin-2/lymphokine activated killer cells (IL-2/LAK) and to characterize the changes in tumor and tumor infiltrating lymphocytes as a result of treatment.

METHODS EMPLOYED

Patients entering this study must have advanced or recurrent metastatic melanoma and tumor accessible to biopsy by minor surgical procedures. Renal, liver, hematologic, pulmonary and cardiac function must be normal. Assessment for eligibility is done in the outpatient clinic. Once eligible, all treatment is conducted in the inpatient unit except for leukapheresis. R24 is administered by continuous infusion for 24 hours beginning day 0. Following the infusion on day 1, IL-2 (4.5 mu/m^2/d) is administered by continuous infusion for 108 hours. On day 7-9, three leukapheresis of 12-15 liters are performed and cells are cultured in vitro with 1000 units/ml of IL-2 for 72-96 hours. On day 11, IL-2 is restarted at a dose of 3 mu/m^2/d for 168 hours. LAK cells are infused just prior to IL-2 on day 11 and again on days 12 and 14. Patients are evaluated for response on day 42. Biopsies are obtained on days 0 (prior to R24), day 11 (prior to LAK), and day 21 (following the completion of IL-2/LAK). Tumor tissue will be tested for R24 binding and antigen saturation, immunophenotyping of tumor infiltrating lymphocytes, expression of tumor-associated antigens and MHC class 1 and 2 molecules, and if possible, expansion and functional characterization of tumor infiltrating lymphocytes. Assays of peripheral blood lymphocytes will include phenotyping, measurement of cytotoxicity against melanoma cell lines, and quantitation of proliferation when stimulated with R24.

MAJOR FINDINGS

Six patients have been accrued at the first dose level (3 mg/m^2/d). Two patients experienced severe pulmonary toxicity (grade 4) with the infusion of LAK cells but did not require intubation. Three patients at this level had progressive disease at their first evaluation, and three patients are too early for response determination. Laboratory correlates are pending.

PROPOSED COURSE

This study will accrue patients until the maximum tolerated dose is reached.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09352-01 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

5FU, Leucovorin, AZT & Persantine for Melanoma, Renal & Colorectal Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigetor.) (Name, title, leboretory, end institute effiliation)*

PI:	D. L. Longo	Associate Director	OAD, BRMP, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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 stenderd unreduced type. Do not exceed the spece provided.)*

The combination of 5FU and leucovorin (LV) has resulted in significant increases in response rates and modest improvements in survival for patients with metastatic colorectal cancer. However, many patients are resistant to treatment at presentation and others acquire resistance during therapy. Resistance may be the result of provision of thymidylate via the alternate salvage pathway from intracellular or extracellular breakdown products of DNA. Persantine inhibits nucleoside transport at the cell membrane thereby blocking salvage of preformed nucleosides. Thus, extracellular nucleosides will be unable to enter the cell while lipid-soluble AZT can diffuse into the cell and compete with any thymidine generated by escape of TS inhibition by 5FU and LV or the intracellular salvage of nucleosides. The combination of these agents should lead to effective thymidine starvation and cell death.

A phase I study using fixed doses of 5FU, LV and persantine with increasing doses of AZT (50 mg, 100 mg and 200 mg) has been completed. Dose-limiting toxicity was not observed and a phase II study is being conducted at the 200 mg dose of AZT. Six patients received AZT at 50 mg, 5 at 100 mg and 5 at 200 mg during the phase I portion of the study. Grade 4 anemia and grade 4 thrombocytopenia were observed in one patient each at 50 and 100 mg dose levels, respectively. No grade 4 toxicity was observed at 200 mg AZT. There was one minor response at 100 mg of AZT in a patient with renal cell carcinoma. Four of the five patients at 200 mg of AZT are not evaluable for response. Thus far, 13 patients have been enrolled on the phase II portion of the study; renal (6), melanoma (4) and colorectal (3).

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI

OBJECTIVES

1. To determine the toxicity of persantine and AZT when added to 5FU and leucovorin.

2. To document any antitumor activity of this combination of agents.

METHODS EMPLOYED

Eligible patients must have histologically documented diagnosis of recurrent or metastatic melanoma or adenocarcinoma of the colon, rectum or kidney. The patient must not have received more than one pior chemotherapy regimen, they cannot have received both 5FU and leucovorin. Patients were evaluated at the Clinical Research Branch, Biological Response Modifiers Program in Frederick. Treatment consisted of 5FU 370 mg/m^2/d for 5 days by intravenous bolus, leucovorin 50 mg/m^2 every 4 hours by mouth for 5 days, persantine 50 mg/m^2 every 6 hours by mouth and AZT (50 mg, 100 mg, 200 mg) every 6 hours by mouth, each for 28 days. The next cycle began on day 29. Five patients each were to receive treatment at AZT 50 mg and 100 mg. If dose-limiting toxicity was not observed, all subsequent patients would be treated at AZT 200 mg. All patients were treated as outpatients. Each patient was treated until a complete clinical remission was obtained plus two additional cycles or until disease progression occurred.

MAJOR FINDINGS

Six patients were treated at the 50 mg dose of AZT. One patient requested to be taken off study after one week of therapy and five completed all planned therapy. Grade 3 nausea, vomiting, and anorexia were seen in one patient. Grade 3 fatigue was seen in one patient. Grade 3 leukopenia was seen in two patients and grade 3 anemia in one and grade 4 anemia in another. No diarrhea was observed. There were no antitumor responses.

Five patients were treated at the 100 mg AZT level. Dose reduction of AZT (50% reduction) and 5FU (25% reduction) was required in one patient because of grade 4 thrombocytopenia requiring platelet transfusions. One patient had grade 3 leukopenia and anemia. No grade 3 nausea, vomiting or diarrhea was observed. One patient had a minor regression in retroperitoneal lymph nodes involved with renal cell carcinoma.

Thirteen patients have been enrolled on the phase II portion of the study. Most patients are currently on treatment and toxicity and response information are being collected.

SIGNIFICANCE

5FU and leucovorin is clearly effective in a minority of patients with metastatic colorectal cancer. However, response rates are only 40%, usually partial and result in only a modest survival benefit. Addition of persantine and AZT has the potential to increase response rates, including possibly complete responses and result in even more prolongation of survival.

There is currently no effective chemotherapy for renal cell cancer and a number of regimens that are of questionable value in metastatic melanoma. Any new effective regimen for either of these tumor types would be important.

PROPOSED COURSE

For colorectal cancer patients, we will initially enroll nine patients at the 200 mg dose of AZT, if zero patients respond we will cease accrual and assume the response rate if our combination is less than 30%. If one response is noted, we will accrue 30 patients. For melanoma and renal cell cancer, 14 patients with each type will be enrolled. If zero patients respond, we will stop accrual and assume that our response rate is <20%. If one response is noted, we will accrue up to 30 patients with that type of tumor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09353-01 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

A Phase I/II Study of a Monoclonal Antibody & IL-2 & Cyclophosphamide

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. E. Janik	Expert	CRB, NCI
	J. W. Smith II	Medical Staff Fellow	CRB, NCI
	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	R. G. Fenton	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Frederick Memorial Hospital (N. Englar); Program Resources, Inc., Frederick, Maryland 21701 (W. J. Urba, J. Beveridge); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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.)*

Previous Biological Response Modifiers Program (BRM) studies (#8705) have shown that endogenous circulating NK and LAK cell activity can be induced and maintained for a prolonged period, using IL-2 twice weekly. In this regimen, IL-2 is given by 24-hour continuous infusion twice weekly for 3 weeks, at a dose of 3 x 107 u/m2 (BRMP units) per 24-hour infusion. Subsequent IL-2 doses are adjusted in the individual patient to sustain high levels of Leu-19 positive cells in peripheral blood, while allowing most treatments to be undertaken in the outpatient setting. Modest evidence of antitumor activity has been seen using this regimen in melanoma. Other studies of a monoclonal antibody, R24, to the GD3 antigen present on melanoma cells, have shown that this antibody given alone results in some antitumor activity in a minority of melanoma patients. Laboratory studies indicate that this antibody can mediate ADCC using circulating LGL cells produced by IL-2. This study was designed to investigate the combination of R24 in a series of escalating dose ranges, together with the IL-2 regimen previously investigated by the BRMP.

PROJECT DESCRIPTION

PERSONNEL

Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Robert G. Fenton	Expert	CRB, NCI

OBJECTIVES

The objectives of this study are:

1. To explore the toxicity of this regimen of R24 in combination with immunotherapy using IL-2 and cyclophosphamide.

2. To determine the immunological effects of the regimen on circulating immune effector cells and, where possible, lymph node and tumor-infiltrating lymphocyte populations.

3. To record the antitumor effects of the regimen in a preliminary patient sample.

METHODS EMPLOYED

Patients are required to have measurable tumor, good performance status (80% Karnofsky or greater), expected survival greater than 4 months, and no brain metastases. Tumor types are restricted to pathologically proven malignant melanoma.

Patients are treated with recombinant IL-2 (Hoffmann-LaRoche) in the following regimen:

3 weeks high-dose induction: IL-2 at 3×10^7 u/m^2 by 24-hour intravenous (i.v.) infusion b.i.w. (inpatient).

Maintenance: IL-2 at 3×10^6 u/m^2 by 24-hour intravenous infusion b.i.w. (outpatient).

Antibody treatment: At fixed doses (according to a Phase I dose escalation scheme) by continuous i.v. infusion over 24 hours in the hospital, twice weekly for a total of 4 antibody doses over a 2-week period. Each MoAB dose will be followed by IL-2 at 3×10^6 u/m^2, beginning 24 hours after the beginning of the MoAb treatment. Treatment with IL-2 without antibody will then continue twice weekly at this dose for 14 additional treatments. These treatments, if tolerated, may be administered as an outpatient.

Patients are assessed for response after 3 months.

Responding patients are retreated with the regimen, after a one-month rest. Responding patients evidencing human anti-mouse antibodies are treated with IL-2 alone.

MAJOR FINDINGS

Nine patients have been accrued to the study and 7 are still undergoing treatment. Two were taken off: one for renal insufficiency and one progressive disease after 3 months treatment. There has been 1 PR (at the 10 mg MoAB dose level) and 1 minor response (at the 5 mg MoAB dose level). Major toxicity appears to be identical with the previously used regimen employing IL-2 alone.

SIGNIFICANCE

This trial is too early for clear-cut analysis. The antibody appears to be tolerated in the doses employed, however, in combination with the desired full dose of IL-2.

PROPOSED COURSE

Accrual will continue. Preparation of humanized versions of two melanoma antibodies are underway: R24 (anti-GD3, the current antibody), and 14.18 (anti-GD2, an antibody to another antigen). Depending on the results of the current trial, additional patients may be studied to define a response rate at what appears to be an optimal dose of R24, and follow-on studies may be designed to employ humanized antibodies in combination with IL-2, as appropriate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09354-01 CRB

RIOD COVERED

ctober 1, 1989 through September 30, 1990

TLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

hase Ib Evaluation of IL-1 Beta

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

I:	R. G. Steis	Medical Officer	CRB, NCI
thers:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	J. J. Oppenheim	Chief	LMI, NCI

OOPERATING UNITS *(if any)*

rogram Resources, Inc., Frederick, MD (W. J. Urba); Cancer Therapy Evaluation rogram, NCI, Bethesda, MD (M. Sznol)

B/BRANCH

linical Research Branch

CTION

STITUTE AND LOCATION

CI-FCRDC, Frederick, Maryland 21701

TAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

HECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

nterleukin-1 (IL-1) is a biological response modifier that has important roles s a mediator in inflammation and in hematopoiesis. Although the ultimate role f IL-1 in cancer therapy may be as a hematopoietic stimulating factor, it has irect antitumor effects that might potentially be exploitable in patients. In his first study of IL-1 beta at the Biological Response Modifiers Program, we re increasing doses of IL-1 beta by half-log increments from a starting dose of 01 mcg/kg given as a daily 15-minute infusion for 7 consecutive days. As of his date, we have escalated the dose to as high as .1 mcg/kg. The toxic effects bserved so far have been fever, chills, nausea, vomiting, and headache. There as been moderate hypotension that required pressors in one patient. One patient eveloped an acute hypertensive episode following her third dose of IL-1 that esulted in acute congestive heart failure with pulmonary edema. As of this ate, three more patients are being accrued to this dose level to attempt to etermine the frequency of this side effect. White blood cell increases up to 0,000 have been observed following IL-1 beta administration. These counts tend o fall during continuing administration of this drug. The white count increase s entirely accounted for by increases in mature granulocytes and bands. More atients will be entered on this study to further characterize the toxic, emopoietic, and immunomodulatory effects of IL-1 beta.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
J. W. Smith II	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
Joost J. Oppenheim	Chief	LMI, NCI

OBJECTIVES

1. To determine the toxicity and maximally tolerated dose of recombinant IL-1 beta given by the intravenous route once a day for seven consecutive days.

2. To determine the immunologic and hematopoietic effects of recombinant IL-1 beta given by this schedule.

3. To determine the effects of indomethacin on the toxicity and the immunologic and hematopoietic effects of IL-1 beta.

4. To determine the pharmacokinetics of intravenously administered IL-1 beta.

5. To note any antitumor effects of the agent.

METHODS EMPLOYED

Patients with refractory solid malignancies are eligible for this study. The starting dose of IL-1 beta is .01 mcg/kg given daily for seven consecutive days. Three patients will be treated at each dose level and dose levels will be escalated by half-log increments. To better define IL-1 beta-related toxic, hemopoietic and immunomodulatory effects at the maximum tolerated dose (MTD), a total of 10 evaluable patients will be treated at this dose level. Because the antitumor effects of IL-1 in animal models can be enhanced by concurrent administration of indomethacin, once the MTD of IL-1 alone is determined, the MTD of Il-1 in combination with indomethacin, 50 milligrams every 8 hours, will be determined starting at two dose levels below the MTD of IL-1 beta alone.

MAJOR FINDINGS

Eight patients have so far been treated with IL-1 beta. Toxic effects observed so far have included nausea, vomiting, fever, chills, and moderate hypotension which required the use of pressors in only one patient so far. One patient developed an acute hypertensive episode shortly after infusion of the IL-1 that resulted in acute congestive heart failure with pulmonary edema. She responded satisfactorily to diuretics and preload reducing agents. Increases in the white blood cell count following IL-1 beta to as high as 20,000 per cubic millimeter

have been observed. All of the increase in white count is accounted for by increases in mature granulocytes and bands. Bone marrow evaluations, which will be performed prior to administration of IL-1 at day 4 and at day 7, have not yet been completed. The immunomodulatory effects of IL-1 beta are being studied but no data are available as yet.

SIGNIFICANCE

If the preclinical studies of IL-1 beta in animals are mirrored by the clinical studies, IL-1 beta will have an important role as a hematopoietic stimulating agent and as a direct antitumor agent. A major difficulty with the use of colony-stimulating factors to hasten marrow recovery following myelotoxic insults is that such agents generally stimulate recovery of granulocyte counts but not platelet counts. IL-1 beta is a stimulator of pluripotent stem cells and in animal studies has resulted in enhanced recovery of both granulocytes and platelets at rates faster than observed following chemotherapy alone. Further, a number of tumor cell types have been studied in animal models in vivo and respond to IL-1 beta treatment, and in some cases with complete responses. If this drug can be tolerated by humans, we plan to proceed with the use of IL-1 beta in conjunction with chemotherapy and possibly with bone marrow transplantation.

PROPOSED COURSE

The MTD of IL-1 alone and IL-1 in combination with indomethacin will be determined with the accrual of additional patients. The data on the immunomodulatory and hematopoietic effects of IL-1 beta will be collected.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09355-01 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

A Phase II Study of CIS-Platinum/IFN-Alfa & IL-2 in Malignant Melanoma

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

PI:	M. Sznol	Principal Investigator	CTEP, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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.)*

High doses of cisplatinum have shown substantial activity in metastatic melanoma. Interferon-alfa is another active agent in this disease and has been shown to potentiate the cytotoxicity of platinum in vitro. Interleukin-2 (IL-2) in combination with interferon-alfa has synergistic antitumor activity in animal models and appears to mediate responses through activation of the host immune system. Since the toxicities of these combinations are non-overlapping and their targets for inducing tumor regression distinct, we combined interferon-alfa/platinum with interferon-alfa/IL-2 in an attempt to improve the therapeutic outcome in metastatic melanoma patients. Interferon-alfa 5 mu/m2 was administered subcutaneously on days 0-3, 7-10, 15-18 and 22-25. Cisplatinum (100 mg/m2 in the first 4 patients, 75 mg/m2 in the next 5 patients) was infused over 30 minutes in hypertonic saline on days 1 and 8. IL-2 was given on days 15-18 and 22-25 by continuous infusion at a dose of 3 mu/m2/d (total 96 hours of infusion for each week). A bolus of IL-2 (3 mu/2) was given prior to the infusion on day 15 and 22, and additional boluses (1.5 mu/m2) were given on days 16-18 and 23-25. Three of 8 evaluable patients responded in adrenal, skin, and lymph node sites. Toxicity was excessive and included intolerable nausea and vomiting, fatigue, creatinine elevation, myelosuppression, confusion, pruritus, and transaminase elevation. Although responses were noted, there was no indication that a high rate of complete responses could be achieved, and toxicity was too severe to justify continued patient accrual.

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Principal Investigator	CTEP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI

OBJECTIVES

To determine the response rate and toxicity of this regimen in patients with metastatic melanoma.

METHODS EMPLOYED

Patients with metastatic melanoma and normal hematologic, liver, pulmonary, and renal function were eligible for this study. All treatment was administered in the inpatient unit. Interferon-alfa 5 mu/m^2 was administered subcutaneously on days 0-3, 7-10, 15-18 and 22-25. Cisplatinum (100 mg/m^2 in the first 4 patients, 75 mg/m^2 in the next 5 patients) was infused over 30 minutes in hypertonic saline on days 1 and 8. IL-2 was given on days 15-18 and 22-25 by continuous infusion at a dose of 3 mu/m^2/d (total 96 hours of infusion for each week). A bolus of IL-2 (3 mu/m^2) was given prior to the infusion on day 15 and 22, and additional boluses (1.5 mu/m^2) were given on days 16-18 and 23-25. Response was evaluated at the end of each 28 day cycle, and cycles were repeated after 1-2 weeks rest.

MAJOR FINDINGS

Nine patients were entered to the study, 7 males and 2 females. The median age was 41 years. Sites of disease included lymph nodes only in 3 patients, subcutaneous plus infiltration of the biceps muscle in 1, skin plus lymph node in 1, lung and lymph node in 1, multiple sites including adrenal or spleen in 2, and one patient with liver and skin metastases from an ocular primary. Six patients had no prior chemotherapy or immunotherapy, one patient had a prior course of IL-1, one patient was previously treated with BCG and DTIC, and one patient had combination chemotherapy.

Eight patients are evaluable for response after completing at least one cycle. One patient has not completed his first cycle at this time and another developed protracted grade 4 nausea and vomiting after the first 2 weeks of treatment but is considered evaluable for response. Three partial responses were seen in the following sites: adrenal and lymph node, lymph node and skin, and skin plus lesions infiltrating the biceps muscle. The first patient achieved partial response approximately 3 months after completing treatment although his lesions were shrinking during two treatment cycles. The second patient is receiving a second cycle of therapy. The last patient was sent home for resection of residual disease. One patient had stable disease 5 months after completing treatment. One patient had marked resolution of ascites and edema with a minor response in a large measurable spleen metastasis, but progressed after the fourth cycle of treatment. Two others had progressive disease.

Toxicity was severe. Grade 3/4 nausea and vomiting were prevalent (5/8) and poorly tolerated by most patients despite reducing the dose of cisplatinum. A grade 3 and a grade 4 creatinine elevation developed following platinum. One patient experienced a grade 3 neurotoxicity during IL-2/interferon-alfa administration. Other toxicities included grade 4 thrombocytopenia and neutropenia, pruritus, hypotension requiring pressors, diarrhea, weight gain, fatigue, myalgias, and grade 3 and 4 transaminase elevations.

PROPOSED COURSE

Although 3 partial responses were seen in the first 8 patients, we feel the regimen is excessively toxic to justify continued accrual. The protocol will be closed after 9 fully evaluable patients are entered. Future studies will focus on combinations of chemotherapy and immunotherapy requiring less hospitalization.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09356-01 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

A Phase I Trial of Concurrent rIL-2 and rIFN-Alfa Administered Subcutaneously

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
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (G. Frey, L. Barnhart)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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.)*

The combination of interleukin-2 (IL-2) and interferon-alfa demonstrated synergistic antitumor activity in preclinical animal tumor models, even at doses substantially below their individual maximum tolerated dose (MTD). Based on these data, we initiated a phase 1 trial of IL-2 and interferon-alfa to determine an optimal regimen for chronic outpatient treatment. Both agents were administered subcutaneously. All patients received IL-2 daily for 5 days each week. The first dose level was 3 mu/m2/d of IL-2, and 2.5 mu/m2 of interferon-alfa thrice weekly. Due to excessive toxicity which required dose reductions early in treatment in 2 of the 5 evaluable patients, the starting dose of IL-2 and interferon-alfa was decreased to 1.5 mu/m2/d, and the interferon-alfa was given daily (rather than TIW) to allow for tachyphylaxis to its systemic toxicities. Although dose-escalation of first IL-2 and then interferon-alfa was attempted, the doses which were tolerated during chronic outpatient therapy were 1.5 mu/m2/d of each agent. A total of 32 patients were accrued to the study, 15 at the MTD. Toxicities included fever, chills, diarrhea, nausea, vomiting, weight loss, hypotension requiring hydration or pressors, and elevations of serum creatinine, bilirubin, and transaminases. One patient developed a neurologic syndrome resembling a focal stroke which resolved. Three partial responses were seen in this study, all in patients with renal cell carcinoma (19 renal cell carcinoma patients entered). We conclude that 1.5 mu/m2 of IL-2 given SQ 5/7 days weekly and interferon-alfa 1.5 mu/m2 administered SQ daily can be given on an outpatient basis to a majority of patients and has activity in patients with renal cell carcinoma.

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Senior Investigator	CTEP, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
William H. Sharfman	Expert	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
John E. Janik	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI

OBJECTIVES

To determine the maximum tolerated dose of interleukin-2 (IL-2) and interferon-alfa administered subcutaneously for chronic outpatient therapy, and to measure in vivo changes in lymphocyte phenotype and cytotoxicity induced by these agents.

METHODS EMPLOYED

Eligible patients included those with malignancies refractory to standard therapy and with normal cardiac, pulmonary, renal, and liver function. Patients were evaluated in the outpatient clinic, and if eligible, were admitted to the inpatient unit for the first week of treatment. All subsequent treatment was administered in the outpatient clinic or was self administered by the patient at home. IL-2 was administered SQ daily for 5 consecutive days each week. Interferon-alfa was administered SQ TIW in the first cohort, and subsequently on a daily basis in every subsequent cohort. Dose levels were as follows for IL-2 and interferon-alfa respectively in $mu/m^2/d$: 3/2.5, 1.5/1.5, 3.0/1.5, 1.5/3.0. An additional 9 patients were placed at dose level 1.5/1.5 to further define the toxicities at the MTD. Immune parameters were drawn on the first and fifth day of IL-2 injection during weeks 1 and 2, on the first day of IL-2 injection of week 3 and the beginning of each 4-week cycle and on the last day of IL-2 injection for each 4-week cycle. Immune parameters included peripheral blood LAK and NK cytotoxicity, phenotyping of peripheral blood lymphocytes, serum levels of soluble IL-2 receptors, neopterin, and beta-2-microglobulin, and antibodies to interferon-alfa and IL-2. Selected patients at each dose level had serum levels of IL-2 and interferon-alfa measured at intermittent time points during the first 24 hours after their first injection.

MAJOR FINDINGS

Thirty-two patients were entered to the study, 21 males and 11 females. Median age was 49. There were 19 patients with renal cell carcinoma; 7 with metastatic melanoma; 3 with a primary non-small cell lung cancer; and one each with endometrial carcinoma, colon carcinoma, and angiosarcoma.

The maximum tolerated dose was established at 1.5 mu/m^2/d of IL-2 and interferon-alfa. Of the 14 evaluable patients at this dose level, 5 were unable to tolerate full doses for 2 months. Dose-limiting toxicities were complete heart block in one patient and hypotension (requiring fluids or pressors) and moderate to severe constitutional symptoms in the other 4 patients. Therefore approximately 64% of patients can tolerate this dose level, with the remainder of the patients requiring dose reduction (to 1 mu/m^2/d of both agents) to complete the course of treatment.

Three partial responses were seen in patients with renal cell carcinoma. Sites of response included liver (in 2 patients), lymph node, and lung. One of the responding patients had previously responded to and then progressed on a sequential IL-2/LAK and interferon-alfa regimen.

Laboratory results are not yet available.

PROPOSED COURSE

Two additional cohorts will be treated with IL-2 alone for the first 4-week cycle at 1.5 and 3.0 mu/m^2/d. After a 2-week washout, these patients will receive IL-2 plus interferon-alfa at the maximum tolerated dose (1.5 mu/m^2/d of each agent). This will allow us to gather additional toxicity data for IL-2 alone and the combination, and will permit a comparison of immune parameters obtained with IL-2 alone versus the combination.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 09357-01 CRB

PERIOD COVERED

October 1, 1989 through September 30, 1990

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

Poly ICLC and Alpha Interferon in Refractory Malignancy

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

PI:	J. E. Janik	Expert	CRB, NCI
Others:	R. G. Steis	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W.J. Urba); Cancer Treatment Evaluation Program, NCI, Bethesda, MD (Mario Sznol)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, 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.)*

This trial was designed to test the combination of Poly-ICLC and alpha interferon in patients with refractory malignancy. Alpha interferon induces the synthesis of a number of interferon responsive genes. These induced gené products are responsible for producing the antiproliferative and antiviral effects of alpha interferon. Two of the well characterized gene products induced by alpha interferon are the 2′, 5′ oligoadenylate synthetase and the P1/EIF2 alpha kinase. Both of these gene products are induced by alpha interferon but require additional factors to activate them. Double-stranded ribonucleic acid is capable of activating both 2′, 5′ oligoadenylate synthetase and P1/EIF2 alpha kinase. Activation of the latter is associated with potent protein synthesis inhibition. The combination of alpha interferon and double-stranded ribonucleic acids has synergestic antiproliferative effects in several human tumor cell lines including lung, bladder, and sarcomas. The levels of synergism range from 3-250 fold. This study will explore the toxicity, immunologic effects and antitumor activity of the combination of alpha interferon and Poly-ICLC administered by daily subcutaneous and 3 x weekly intramuscular injection, respectively. To date, seventeen patients have been entered on the trial. Two patients were removed from study during the initial week of therapy with alpha interferon alone, one patient due to Grade IV hepatic toxicity and the second patient due to Grade III fatigue. There have been three responses to date, a partial response in a patient with malignant melanoma and two minor responses, one in a patient with renal cell cancer and a second in a patient with rectal carcinoma.

PROJECT DESCRIPTION

PERSONNEL

John E. Janik	Expert	CRB, NCI
Ronald G. Steis	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Senior Staff Fellow	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Robert G. Fenton	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI

OBJECTIVES

1. To determine the maximal tolerated dose of Poly-ICLC given in combination with interferon alpha.

2. To determine whether interferon alpha induces synthesis of double-stranded ribonucleic acid inhibitor in peripheral blood mononuclear cells and in tumor tissue.

3. To determine whether Poly-ICLC will activate the double-stranded ribonucleic acid inhibitor and to determine the optimal concentration of Poly-ICLC necessary for activation.

4. To determine in a preliminary fashion the antitumor effects the plasma pharmacokinetics of Poly-ICLC and interferon alpha used in combination.

METHODS EMPLOYED

Patients entered on this trial must have a documented histologic diagnosis of a malignancy that is refractory to standard therapy. They must have a Karnofsky performance status of 70% or greater, a life expectancy of at least three months, evaluable or measurable disease and adequate physiological function. Patients with brain metastases are eligible for this trial if the metastases have been controlled by radiation therapy and/or surgery. This is a Phase I trial of the combination of interferon alpha and Poly-ICLC. Patients will receive interferon alpha daily as a single subcutaneous injection. During the second week of therapy, patients will begin treatment with Poly-ICLC administered as an intramuscular injection 3 x weekly on Mondays, Wednesdays, and Fridays. The treatment will be continued without dose adjustment for one month. Interferon alpha will be administered at one of three dose levels to individual patients - 1, 3, or 10 million units/m^2. Poly-ICLC will be administered at doses of 0.1, 0.5, 1, and 3 mg/m^2. Five patients will be treated at each dose level of the combination of Poly-ICLC and interferon alpha. The initial month of therapy will be used as the period to determine the maximum tolerated dose of the combination.

MAJOR FINDINGS

The first two dose levels of the protocol, the combination of interferon alpha at a dose of 1 million units/m^2 and Poly-ICLC at 0.1 mg/m^2 and interferon alpha 3 million units/m^2 and 0.1 mg/m^2 of Poly-ICLC have been completed. The third dose level 10 million units/m^2 of interferon alpha and 0.1 mg/m^2 of Poly-ICLC is accruing patients. Seventeen patients have been entered on the trial to date. Two patients have been removed from therapy during the initial week of therapy with interferon alpha alone. One patient at the 3 million unit/m^2 dose level was removed for Grade IV hepatic toxicity and one patient at the 10 million unit/m^2 dose of interferon alpha was removed from study due to Grade III fatigue. No unexpected toxicities have been noted with use of this combination of drugs. The toxicity at the lowest dose level of Poly-ICLC 0.1 mg/m^2 does not seem to be accentuated over that which would be anticipated with the use of interferon alpha alone. Seven patients with colon cancer, three patients with melanoma, two patients with renal cell carcinoma, and one patient each with breast, neuroepidermal and rectal carcinoma have been treated on this study. Three responses have been seen with a minor response in a patient with renal cell carcinoma at the lowest dose level, a partial response in a patient with melanoma at the second dose level, and a minor response in a patient with rectal carcinoma at 10 million units/m^2 of interferon alpha and 0.1 mg/m^2 of Poly-ICLC. At this time, the study is on hold due to problems with the supply of Poly-ICLC.

PROPOSED COURSE

We plan to treat additional patients as outlined above in order to complete this Phase I trial within the coming year.

SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1989 - September 30, 1990

I. Introduction and General Organization

The Developmental Therapeutics Program (DTP) is the preclinical unit within the Division of Cancer Treatment charged with the discovery and development of new anticancer and anti-HIV agents for introduction into clinical trials. The DTP utilizes both intramural and extramural (grants and contracts) mechanisms to accomplish its mission. The Program is currently comprised of nine extramural branches and four intramural laboratories organized under the Associate Director.

Over the past year, there have been major organizational and personnel changes. Dr. Michael Boyd, the former Associate Director of the Program, elected to return to more direct laboratory investigation. Dr. Boyd has been appointed the Laboratory Chief for a new intramural unit called the Laboratory of Drug Discovery Research and Development. The temporary assignment of Dr. Michael Grever, Deputy Director of the Division of Cancer Treatment, to be the Acting Associate Director of DTP has been effective since February, 1990. Currently, the position of the Associate Director for this Program is being advertised for competition.

The Laboratory of Drug Discovery Research and Development was established as an essential resource for the Program. This group of intramural investigators is dedicated to the expeditious development of high-priority agents which have been identified as being of substantial promise for the treatment of either cancer or HIV infection. In this sense, the laboratory represents a combination of both applied and basic research capabilities that can be rapidly mobilized to address high priority problems that otherwise might delay a promising drug getting to clinical trial. Furthermore, the laboratory is dedicated to the development of prototypic compounds that will maximize the drug discovery and development processes already operational within DTP. Finally, the natural product drug discovery effort represents a major focus for investigators within this laboratory. In order to accomplish these objectives, the laboratory will be divided into three sections each under the leadership of a senior investigator: Cell Biology Section (Dr. Boyd); Natural Products Section (Dr. Cardellina); and Pharmacolgy and Metabolism Section (Dr. Malspeis).

The Antiviral Evaluations Branch (AEB) under the direction of Dr. John Bader has been approved constituting the most recent and the ninth extramural branch within the Program. This branch is responsible for overseeing the actual operation of the anti-HIV screen, the interpretation of the screening data, and the generation of reports to the suppliers. Furthermore, the professional staff of this branch assume the major responsibility for the secondary testing necessary for the positive leads generated from the screening effort. Dr. Bader is the chairperson for the Biological Evaluations Committee for antiviral agents (BEC/AIDS) which represents the interdisciplinary group of investigators responsible for coordinating the studies needed to bring promising new leads to the Decision Network Committe of the Division of Cancer Treatment.

The Laboratory of Biochemical Pharmacology has been incorporated into the Laboratory of Medicinal Chemistry under the overall leadership of Dr. John Driscoll. Dr. David Johns, however, will continue to be actively involved with other investigators from the original laboratory. The consolidation of these intramural laboratories will enhance the research capabilities of both units in accord with the recommendations of the respective site visiting teams.

Major personnel changes (in addition to those described above) include: the recruitment of Dr. Louis Malspeis, former Professor of Pharmaceutical Chemistry at the Ohio State University College of Pharmacy, as a section leader in the new intramural Laboratory of Drug Discovery Research and Developmemt; and the departure of Dr. Charles Grieshaber, Chief of the Toxicology Branch, to the Food and Drug Administration. Dr. Joseph Tomaszewski has been appointed Acting Chief of the Toxicology Branch. Dr. Theodore McLemore has also left the National Cancer Institute for a position in Texas.

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 (OAD)

In addition to the administrative changes which have been briefly described above, there have been organizational changes within the Program that hopefully will expedite both the drug discovery and developmental processes:

(1) Discovery and Development of Anticancer Agents-

While the anti-HIV screen and drug evaluation programs have been efficiently operating for the past year, the anticancer screen has been fully operational for approximately five months. Extensive efforts to standardize the current anticancer screen prior to testing unknowns and to develop a satisfactory mechanism and format for reporting the results to the respective suppliers of compounds were established.

On April 1, 1990 the full effort of the anticancer screen was devoted to screening known chemical structures. This screen is now operating at approximately 300-400 tests per week in an effort to decrease the backlog of chemical compounds which have been previously submitted, and to identify promising lead compounds that will warrant further testing. The current decision to examine compounds with known chemical structures in the screen will increase the likelihood of getting promising agents to the clinic in the near future. However, we are firmly committed to fully explore natural products as promising anticancer leads. As the backlog of submitted structures is reduced, screening of natural product extracts will resume. We estimate that resumption of natural product extract screening will be implemented by the late fall of 1990. Subsequently, the Acquisition Input Committee chaired by Dr. Narayanan, Chief of the Drug Synthesis and Chemistry Branch, will balance the effort between compounds of known chemical structure and the natural product extracts.

Since April, 1990 there have been over 3,000 supplier reports mailed. There have been approximately 46 compounds identified by the Cancer Screening Committee as demonstrating sufficient interest from the screen to be recommended for further testing. We have formally identified a Cancer Screening Committee under the supervision of Dr. Boyd that is responsible for monitoring the progress of the anticaner screen and for selecting the agents demonstrating sufficient interest to be passed onto the Biological Evaluations Committee for anticancer agents (BEC/Cancer). Dr. Plowman serves as the chairperson for this extremely important committee which is responsible for steering the process of secondary in vitro and in vivo studies which are necessary to prepare an agent for presentation to the Decision Network Committee. The interdisciplinary membership of this committee meets every two to three weeks to carefully consider all aspects of drug development ranging from the uniqueness of the chemical structure to the potential difficulties that might be encountered with drug production and formulation. The

responsibility of this group (i.e., BEC/Cancer) is to provide a comprehensive plan for testing and to track the results in an efficient manner through the point of presentation to the Decision Network Committee.

After an agent has been approved by the Decision Network Committee for further development, the tracking of its progress becomes the responsibility of the Operating Committee for DTP. There are separate Operating Committees for both anti-HIV agents and anticancer agents. Both of these committees are now chaired by Dr. Grever in his capacity as the Acting Associate Director. A new approach to the developmental process for anti-HIV and anticancer agents has been instituted over the past six months. Those agents which are identified as being high-priority are assigned to a specific individual within the Program, and a special Working Group of interdisciplinary investigators is assigned to each project. In contrast to the special working groups which were informally convened by DTP staff in the past, the current Working Groups must meet at regular intervals and provide progress reports to the Associate Director at bi-monthly intervals. Furthermore, the chairperson of each group must be responsible for the agent until it formally enters phase I clinical trial in patients. The current list of Working Groups and the responsible chairperson is provided in Table 2.

(2) Extension of Program Capabilities in Drug Discovery-

As a complement to the anticancer screening effort, DTP established a National Cooperative Drug Discovery Group Program (NCDDG) in 1983. This effort exploits recent developments in biomedical research for the discovery of innovative treatment for cancer. Multidisciplinary and multiinstitutional 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 utilizing novel models which will more accurately predict the efficacy of research efforts. Currently, there are eleven funded groups and twelve more have been approved for funding. Approximately $15,000,000 is earmarked for this important aspect of the program. These new awards will greatly expand efforts to discover new anticancer agents from natural sources, such as the tropical rain forests and marine habitats, and will stimulate diverse types of research projects to find new therapies using three general approaches: general mechanism of action, specific disease-oriented strategy, and novel model development.

The NCDDG Program has been successful in bringing several new therapies to clinical trial. Four agents reported last year for the first time are still undergoing active clinical investigation through other support mechanisms, and a fifth product of this program has entered clinical trials over the past year. One of these products (a novel diphtheria toxin-related interleukin-2 fusion protein) has produced several responses in previously treated cancer patients, and was presented to the last Decision Network Committee. Therefore, DTP has established a balanced program which encompasses both the empiric screening efforts and the rational approaches to drug design and discovery.

Over the past two months, DTP staff have initiated an effort to examine the mechanism for making the resources of the Natural Products Repository available to other investigators who are committed to discovery of effective agents for the treatment of cancer and viral illnesses. Providing access to material within this repository is complex. A careful examination of the scientific proposals for utilization of the material, and an assessment of the capabilities for following through on the proposal are necessary. Furthermore, protection of the rights of the suppliers of the material is also necessary.

The cost and effort expended in the procurement and characterization of the material within the repository mandate careful scrutiny of the proposed utilization to avoid depletion of this extremely valuable resource. Efforts are needed to avoid duplication of research activities, yet to maximize the potential for new drug discovery. A committee will establish the procedures for submitting a research proposal to gain access to the Natural Products Repository, and following review will determine if the proposal has sufficient scientific merit and insure that the rights of the supplier will be honored. Thus, a coordinated utilization of this resource will actually extend the research capabilities of DTP.

(3) Progress Report of the Program Development Research Group (PDRG)-

The mission of this group of investigators was to develop research projects that would support the new drug discovery and development programs for the treatment of cancer and AIDS. Studies included projects to further refine the screening models and assay methods used in the cancer and AIDS screens. Model development was pursued to identify active new agents deserving further effort. Furthermore, extensive biochemical analysis of human tumor cell

lines and fresh human tumors were focused on prostanoid biosynthesis, metabolic activation of Ipomeanol, and the molecular genetic approaches to elucidating the human lung cytochrome P450 hemoprotein monooxygenase system.

(a) Cancer Research Projects: PDRG investigators had completed the development and evaluation of a rapid, sensitive, and inexpensive method for measuring the cellular protein content of adherent and suspension cultures in 96-well microtiter plates. This assay procedure was adaptable to the enormous anticancer drug screening effort. The basis for this assay resided with the ability to utilize a computerized system of data procurement and analysis which determined the protein bound dye, sulforhodamine B (SRB). The laboratory demonstrated that determinations of the SRB assay are linear with cell number and protein content. This assay was superior to both the Lowry and Bradford assays of cellular protein content, and compared favorably to assays of 19 other visible dyes. The SRB assay provided a colorimetric end point that is non-destructive, indefinitely stable, and invisible to the naked eye. It provides a sensitive measure of drug-induced cytotoxicity, quantitated clonogenicity, and was suited to high-volume, automated screening.

Recently, the PDRG staff in collaboration with the extramural DTP staff and Program Resources, Inc. (PRI) scientists extensively compared the SRB assay to another tetrazolium-based assay (MTT). Extensive testing of 243 compounds at five dose levels were examined using both the MTT and the SRB assays againt 38 tumor cell lines. Additional retesting was also conducted in a limited number of cell lines to determine the reproducibility. The results demonstrated that under the experimental conditions employed and within the limits of the data analyses, the assays performed quite similarly; however, the SRB assay had superior practical advantages for large-scale screening.

Furthermore, the specific activity of the MTT assay was demonstrated to be highly dependent upon maintenance of cellular concentrations of the reduced pyridine nucleotide concentrations within the tumor cells.

The growth and drug sensitivities of the panel cell lines were examined, and the colon cell line panel had the most rapid growth. The leukemia/

lymphoma cell line panel had the next most rapid growth. In general, the leukemia/lymphoma panel appeared to be the most sensitive panel, and the colon cancer panel was relatively resistant to chemotherapy.

Cancer Drug Screen Model Devlopment: PDRG investigators developed a simple method for constructing three-dimensional colonies attached to plastic. These colonies can grow under these conditions for weeks yeilding a multi-layered, macroscopic colony. Drug efficacy is measured as the regression or the extinction of the preformed colonies. The chemosensitivity frequently differs in this assay from that observed in subconfluent cultures. These three-dimensional colonies may provide a superior model of solid tumor architecture that provides some resemblance to the in vivo situation with respect to spatial considerations for cell growth.

A rapid in vitro method for evaluation of potential antitumor drugs that require hepatic metabolic activation was developed. Although many methods have been published, practical assays adaptable to large-scale screening have been lacking. PDRG investigators found that incorporation of a liver subcellular fraction (S9) into an established cell growth inhibition assay significantly increased the cytotoxicity of cyclophosphamide. This method also proved suitable for studies of metabolic detoxification by conjugation reactions.

A method was developed to support the proliferation of primary cultures of alveolar type II cells from the rat lung. In addition to defining the conditions necessary to promote the proliferation of these "normal" cells in vitro, confirmation that the proliferating cells were actually alveolar type II cells was accomplished using microcinematography, tannic acid staining for intracellular inclusions, intermediate filament analysis, determination of phagocytic activity, and electron microscopic studies. Furthermore, a computerized system was developed for the soft agar culture assay that will be useful for drug sensitivity analysis.

Demonstration of Selective Cytotoxicity of Pseudomonas Exotoxin Coupled to an Antibody Directed Against an Ovarian Cancer Cell Line: Pseudomonas exotoxin coupled to a monoclonal antibody raised against a human ovarian cancer cell line was tested for differential cytotoxicity against 56 cell lines derived from human cancers of the breast, central nervous system, colon, lung, ovary, kidney, and melanoma. Reaction of the monoclonal antibody with each cell line was determined immunocytologically. A high degree of selective toxicity was detected against the mucinous adenocarcinoma cell lines derived from the ovary, colon, breast, and to a lesser extent by the lung.

Extensive biochemical characterizations of human tumor cell lines, fresh human tumors, and normal tissue counterparts were also performed. Prostanoid biosynthesis may be characteristic of certain histologic classes of human tumors, in particular that of non-small cell lung cancer. The distribution of PGH synthase activity was characterized. The activity was most often demonstrated in lung cancer cell lines. The activity was highest in non-small cell lung cancers, and undetectable in nine small cell lung cancer cell lines.

Metabolic activation of Ipomeanol, a novel anticancer agent with promise of activity in lung cancer, was measured using a covalent binding assay of the radiolabeled drug. The activation was examined in 18 human cancer cell lines, normal lung tissue, and in 56 samples of lung cancer obtained from patients. There was a wide interindividual range of Ipomeanol metabolism in both the normal and the lung cancer cells/tissues.

Molecular Biology Studies in Lung Cancer: Since P450 cytochrome is involved in the metabolic activation of procarcinogens, extensive investigation of the following genes were performed: 1A1; 2B1; 2D1; 2F1; and 4B1. In 75 lung cancer patients, altered regulation of 1A1, 2B1, 2F1, 4B1 were demonstrated in comparison with normal lung tissue. In general, there was a decreased expression of 4B1 in lung cancer tissue; 2B1, 2F1, and 2D1 were not expressed in lung cancer; and, 1A1 had altered expression.

The expression of CYP1A1, which is the major polycyclic aromatic hydrocarbon inducible cytochrome presumed to be important in pulmonary carcinogenesis and toxicology, was examined in 56 lung cancer specimens. While the expression of this gene is demonstrable in 89% of smokers, it was undetectable in the non-smokers. Detectable m-RNA levels were demonstrated in 47% of the tumors from the smokers. Furthermore, there was an apparent alteration of expression of the mRNA species observed in 50% of the lung cancer patients.

Finally, investigation of CYP4B1, which is an apparent lung-specific P450 gene, was examined. There was a 20 fold variation in the level of expression, but it was expressed in all samples of normal lung. In contrast, 44% of the lung tumors (8/18) expressed the CYP4B1. In all cases of the lung cancer, the level of expression was approximately 20% of that in normal lung tissue. The expression of this gene in the lung cancer specimens was primarily observed in the adenocarcinoma histologic sub-types.

(b) Antiviral Research Projects

The antiviral drug discovery and development program has been successfully launched with close collaboration of intramural investigators and extramural investigators from the Office of the Associate Director and Program Research, Inc. Recent achievements include:

Anti-HIV Drug Activity In Vitro: Impact of Method of Infection and Virus Infectivity: The in vitro screen for anti-HIV agents is based on virus-induced cytolysis of T-lymphoblastoid CEM-SS cells. Over the past year, investigators have demonstrated that antiviral activity in this assay was influenced by: the quantity of virus input; status of viral replication at the time of drug addition; method of infection of the target cells; and the temperature and time of injection with respect to addition of the drug. Therefore, conclusions pertaining to the antiviral activity of a drug in the assay must give consideration to these factors.

Development of a Semi-automated, Multiparameter Assay for Anti-HIV Drug Screening: PDRG investigators have developed a semi-automated multiparameter assay which addresses a full-range of criteria by which to characterize the activity of antiviral agents against T-lymphoblastoid cells either acutely or chronically infected with HIV. Concurrent with the metabolic tetrazolium assay, cell viability, total DNA content supernatant reverse transcriptase activity, p24 core antigen production, and the synthesis of infectious virions can be assessed from a single well from the 96-well microtiter plate.

Interaction Laser Cytometric Analysis of Retroviral Protein Expression in HIV-infected Lymphocyte Cell Lines: PDRG investigators have developed a method to examine the expression of HIV envelope glycoproteins gp 160, gp41, gp120, and the core protein p24 in the HIV infectable cell lines H9, CEM-SS, and C8166. This method allows for the ultrasensitive detection of fluorescence signals at the single cell level and, when combined with specific anti-HIV antibodies, permits unique quantitative detection of HIV antigens.

Mechanistic Studies on Oxathiin Carboxanilide, A Novel Potent Inhibitor of HIV Reproduction: Oxathiin carboxanilide, which was highly active in the anti-HIV screen, has been extensively investigated within PDRG. The compound inhibits virus reproduction, infectious virus, and extracellular viral p24 antigen. The point of action differs from that of the active nucleosides, and late addition with continued treatment appears to effectively prevent virus-induced effects.

Cellular Microencapsulation Technology for the Evaluation of Anti-HIV Drugs In Vivo: PDRG investigators investigated the feasibility of an assay that involved the microencapsulation of human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV. The encapsulated cells would then be implanted into athymic nude mice and recorded after drug treatment in vivo. The demonstration that the invasion of mouse immune cells could not be overcome in the

assay led PDRG investigators to conclude that this technique was not feasible unless the immunogenic reaction in the mice could be inhibited.

(4) Antiviral Evaluations Effort

This group of extramural scientists has recently been formed into the Antiviral Evaluations Branch. Dr. John Bader is the Branch Chief, and is responsible for coordinating the drug discovery and developmental efforts in the treatment of AIDS and other fatal viral infections.

This group of extramural scientists under the Office of the Associate Director of the DTP established a very effective unit for the screening and further evaluation of anti-HIV drugs. Standardization and further refinement of the primary in vitro anti-HIV drug screen continued, and the large-scale screening capacity progressively increased. As of May, 1990, approximately 90,000 screening tests had been performed. Over 16,000 unique synthetic compounds and 15,000 extracts from biological materials had been tested as of May, 1990. In addition, over 6,000 fractions from active biological extracts had been further examined. From these tests, approximately 250 pure compounds and 300 extracts had been found to be active. Among the active pure compounds, new members of seven already-known active anti-HIV chemical classes were represented. More significantly, new active lead compounds and related derivatives have been discovered in fourteen chemical classes not heretofor associated with anti-HIV activity. Development and implementation of confirmatory tests for anti-HIV activity, i.e., testing for inhibition of virus production as well as inhibition of virus-induced cell killing, have become standard. Also, protocals which allow testing of active compounds for range of action and determination of mechanism of action have been implemented. In addition to a compound's activity in the primary screen, testing in other cell lines and against other viruses (e.g., HIV2) are performed. Collaborative experiments focusing on the stage of virus reproduction affected are accomplished. These latter studies are designed to provide valuable information relevant to the development of the compound for eventual clinical use.

Intramural Laboratories of the Developmental Therapeutics Program-

B. Laboratory of Molecular Pharmacology (LMP)

(1) DNA as a Target for Chemotherapy: The major goal of this laboratory is to obtain knowledge that could be applied to the development of new drugs and strategies for the selective killing of human tumor cell types. This laboratory has developed substantial data regarding the relationship of the topoisomerases as targets for chemotherapeutic agents.

During the past two years, efforts have focused on defining the factors and mechanisms which govern how drug-induced DNA-topoisomerase lesions produce cell death. For example, calcium depletion has been demonstrated to protect against the cytolethal consequences of certain drug-induced DNA-topoisomerase lesions. Furthermore, DNA-topoisomerase lesions produced by doxorubicin appear to be located in the nuclear matrix-associated regions.

The base sequence requirements for interaction of the topoisomerase inhibitors are being extensively pursued. Doxorubicin-induced cleavage sites invariably have an A as the first upstream base relative to at least one of the two breaks produced by the enzyme molecule in the DNA duplex. There is an unusually strong reaction site induced by amsacrine at a c-myc promoter. The modification of a drug's structure to enhance selectivity in the interaction with DNA is the ultimate objective in these experiments.

The molecular mechanism of action of the alkylating agents continues to be an important target of research in this laboratory. The dependence of the base sequence relative to the selective cytotoxicity is being correlated with the length of the connecting chain and the mono or bifunctionality of the alkylating agents. The monofunctional compounds are extremely potent, do not form covalent crosslinks, and are a unique mechanistic class of drugs.

A major new finding is that the DNA sequence selectivity of intercalating mustards is affected in a systematic way by the length of the connecting chain. Furthermore, examination of intracellular pharmacokinetics of several common nitrogen mustards has demonstrated differences in

the rates of formation and removal of DNA crosslinks which may explain the differences in sensitivity to these agents.

Since cell proliferation requires a coordination of both DNA and histone syntheses, the selective uncoupling of these events in neoplastic cells could provide a potentially novel approach to the treatment of cancer. The unique stem-loop structure of most histone mRNA's may regulate degradation of the mRNA by a specific nuclease. Oligonucleotides that mimic the stem-loop sequences might successfully uncouple the histone and DNA syntheses. Furthermore, studies are focusing on the H2A family of histone proteins. While synthesis of the major H2A proteins is synchronized with DNA synthesis, synthesis of several of the specific proteins within this family are not linked to the DNA. Differences in the ratios of mRNA forms between malignant and normal cells have also been demonstrated.

(2) DNA Damage and Repair: The studies of preferential DNA repair in active genes has been directed towards the effects of chemotherapeutic agents in addition to that produced by UV light and carcinogens. Several new observations include: (a) nitrogen mustard adducts form to differing extents in different genomic regions, and are repaired preferentially; (b) in a comparison of two human ovarian cancer cell lines of differing drug sensitivity, the cisplatinum-induced adduct repair in the DHFR gene was similar in the two lines, but the DNA interstrand crosslink removal was slower in the sensitive line; (c) UV-induced damage was more efficiently repaired in a resistant cell subset from a panel of plasmacytoma cell lines; and (d) gene specific repair of UV-induced damage was inhibited in CHO cells in the presence of inhibitors of both topoisomerase I and merbarone (reported to inhibit topoisomerase without inducing cleavage complexes). In contrast, there was no significant inhibition of the DNA repair in that system with either agent used alone. The laboratory is currently exploring the role of gene-specific repair in multi-drug resistance.

Work is also being done on the isolation and characterization of genes whose expression is induced by cell injury by agents such as ultraviolet light, heat, and chemotherapeutic agents. The mechanism of subsequent cell death following the induction of genes responsive to DNA

damage is being explored. Twenty different cDNA transcripts induced by DNA damage have been isolated and sequenced. In addition, a large collection of mammalian cDNA clones for heat-shock genes have been isolated.

In resistant Burkitt's lymphoma cells, an increased expression of one of the "gadd" (growth arrest and DNA damage inducible) genes and another gene for DNA polymerase beta (putative DNA repair polymerase) have been demonstrated. Furthermore, the gene for O^6-alkylguanine-DNA alkyltransferase was cloned, and found to be underexpressed in human tumor cells with increased sensitivity to nitrosoureas. Further specific investigations of the DNA-damage inducible genes are being pursued in an effort to discover potential therapeutic leads.

(3) Tubulin as a Site for Pharmacologic Attack: Tubulin, which is the major protein in the microtubular structures of the mitotic spindle apparatus and of the cytoskeleton itself, is the target of action of the vinca alkaloids and the novel agent, Taxol. This new area of investigation for this laboratory is exploring the potential utility of new types of tubulin binders. An examination of the biochemistry and the structure-activity relationships of this exciting class of agents may provide many new interesting leads (e.g. new natural products of interest include Combretastatin and the Dolostatins; and, the observation that certain nucleotides can replace GTP in tubulin polymerization with a resultant abnormal polymer resembling those formed in the presence of Taxol).

C. Laboratory of Biological Chemistry (LBC)

This laboratory has identified the cellular reactions, which are critical for proliferation and differentiation, as the targets for drug design. Interest in the key biochemical events which actually signal either cell proliferation or differentiation, and in the protein modification reactions that are critical to the biologic activity of specific cellular proteins represent the major research projects of this laboratory.

(1) Protein Modification Reactions: Protein modification reactions may determine the intracellular location and proper functioning of key proteins. Thus, it may be possible to alter the activity of oncogene products or other

important proteins by interfering with the localization in the plasma membrane or other cellular compartments. Investigators within the LBC are exploring the post-translational protein acylation with myristic acid (i.e., N-myristoylation),isoprenoids (i.e., isoprenoylation), and retinoic acid (i.e., retinoylation) as potential new targets for chemotherapeutic drug development. Both isoprenoylation and myristoylation play key roles in targeting the cytoplasmic onc-kinases or retroviral gag structural proteins to the inner plasma membrane cell surface.

N-myristoyl transferase (NMT) from cow brain was purified and characterized in this laboratory. A 52 Kda NMT isoenzyme subunit has been purified sufficiently for microsequencing and antibody production which will aid in the development of probes for screening for the NMT gene in cow brain cDNA libraries. Several new compounds have been identified as in vitro NMT inhibitors, and N-myristoyl tetrazole inhibits N-myristoyl coenzyme A synthetase both in vitro and in vivo. Both of these enzymes are important in the N-myristoylation pathway.

Investigators in this laboratory have also prepared cDNAs of the HIV p17gag and the p27nef genes which encode N-myristoylated proteins. Utilizing bacterial and mammalian vectors, these genes have been expressed in both E. coli and cos cells, the resultant purified p17gag protein was N-myristoylated in vitro with cow brain NMT, and is being used as a probe to identify specific N-myristoyl-gag membrane "acceptors".

Isoprenoids are required for a variety of cellular biological functions including proliferation, membrane structure and function, cell adhesion, and cytoskeletal functions. While the function of isoprenylation is not well-understood, a subset of important cellular proteins (members of the ras oncogene and G-protein families) is modified by the covalent attachment of long chain isoprenoids (e.g., farnesyl, geranylgeranyl) to the cysteins located close to the carboxyterminus of the protein. Since farnesylation is obligatory for the transforming activity of ras oncogenes, the efforts in the laborarory to characterize the post-translational modification may provide a target for anti-neoplastic drug development. The cDNA of a gene which has been demonstrated to be essential for post-translational maturation of p21

ras and for its subsequent binding and transforming activity is being investigated for its farnesyl transferase activity.

The requirement for isoprenylation in the regulation of cell shape and adhesion is being investigated at the cellular level in epithelial cells. Since isoprenoids are derived from mevalonic acid, the demonstration that depletion of the cellular mevalonate results in changes in the cell-cell adhesive contacts and in tubulin organization are important. Methods to resolve isoprenoids from other cellular lipids and to assay isoprenyltransferase have been developed, and will be utilized for further biochemical investigation.

Recent clinical results have demonstrated the importance of retinoic acid in the treatment of human leukemia This laboratory has been involved with gaining basic knowledge on the process of terminal differentiation, the mechanism of action of inducers, and finding clinically useful combinations of inducers. While many of the biologic effects of retinoic acid have been described, the mechanism of action is unknown. Investigators in this laboratory have demonstrated that a covalent bond is formed between retinoic acid and protein. In HL60 there is only one major retinoylated protein species, and it is located in the nucleus. This retinoylated moiety is probably linked via a thio-ester bond, and occurs at very low level concentrations of retinoic acid. Evidence exists that this low-level retinoylation has a functional role.

(2) GTP-Binding Proteins: The emerging family of structurally related proteins (i.e., small GTP-binding proteins) has been implicated as regulators of a diverse array of important cellular functions including: cell transformation and growth, protein synthesis and processing, targeting of membrane vesicles and organelles, and activation of secondary messenger systems (e.g. phospholipase C). While all members of this class of proteins may share similar mechanisms of cellular regulation, this laboratory has focused on the ARF proteins (i.e., ADP-ribosylation factors). The ARF proteins in mammalian cells are critically important for protein secretion, and the role of ARF in regulation of growth factors is being actively pursued. Specific functional domains of the ARF proteins are being mapped with

both point and deletion mutations, as well as antibody probes. Peptide inhibitors of ARF have been constructed, and are being investigated in vitro to determine the role of ARF in protein secretion. These efforts, hopefully, will eventually result in new targets for chemotherapeutic intervention in the secretion of protein growth factors.

(3) Pyrimidine Nucleotide Synthesis: Furthermore, elucidation of the sequence of events between growth factor-receptor interaction and the activation of the obligatory pathways may yeild an increasing number of potential targets for drug modulation. Activation of uridine uptake is an early event in the mitogenic response. In addition, early activation of pyrimidine nucleotide synthesis is linked to the synthesis of extracellular matrix (e.g., hyaluronate). Since hyaluronate has been implicated as a factor involved in tumor invasion and metastasis, elucidation of the regulatory factors involved with the synthesis of the extracellular matrix could be useful for the discovery of agents with anti-invasive or anti-metastatic properties. Factors capable of increasing the uridine uptake and UDP-glucuronide are: EGF, PDGF, IL-1 and phorbol esters. Certain growth factors stimulate fibroblasts to secrete hyaluronate, and recently the BT-20 human breast cancer cell line conditioned media was demonstrated to stimulate hyaluronate synthesis by fibroblasts. Thus, intercellular communication between the neoplastic and normal cells had been established. Investigation to define the biochemical mechanisms involved with the mitogen-stimulation of hyaluronate indicated an increase in the UDP-glucose dehydrogenase activity following mitogen activation. Since UDP-xylose is a potent inhibitor of this enzyme, the composition of the exracellular matrix may be amenable to modification.

The relative contribution of de novo and salvage synthesis to tissue pyrimidine nucleotide pools is an important parameter in designing anti-pyrimidine therapies. Appropriate methods were not previously available to determine the relative contributions of these pathways in vivo. Using stable isotopes, GC/MS methodology, and a novel method of data analysis, the de novo synthesis in murine tumors and tissues was determined. In the tumors, the de novo synthetic pathways were relatively more important. In contrast, in the

normal tissues, there was variation in the relative importance of either pathway.

Studies on the cellular pharmacology of DUP-785, a new anti-pyrimidine antitumor agent, demonstrated that there is a direct correlation between inhibition of the de novo pyrimidine synthesis, changes in pyrimidine nucleotide concentrations, and cell proliferation following short-term drug exposure. In contrast, prolonged exposure (i.e., >24 h) results in a failure of cell proliferation despite restoration of normal pyrimidine nucleotide pools. Clinical protocal design may be affected by the duration of drug exposure times.

Studies have been conducted to characterize the P-glycoprotein phosphorylation and the associated Ca++ and phospholipid-dependent protein kinase C activities in multidrug resistant cells. The overexpression of PKC is closely associated with the mdr phenotype in both leukemic and breast cancer cell lines.

In addition, data generated in cell lines with resistance to doxorubicin has suggested that phosphorylation of P-glycoprotein may be an important pharmacologuc target for reversing the multidrug resistant process, protein kinase C may modulate the level of resistance to some anticancer drugs.

D. Laboratory of Medicinal Chemistry (LMC)

The discovery and development of new anticancer and antiviral drugs are the major objectives for this group of investigators. Organic, analytical, and peptide chemistry is utilized in addition to molecular modeling to accomplish the objectives.

Significant preclinical analytical work has been completed in support of the planned clinical trial of cyclopentylcytosine (CPE-C), which is a cytidine analogue of the natural product neplanocin. Furthermore, the total synthesis of neplanocin F has now been accomplished. In addition, 3-Deazaneplanocin A, a compound previously synthesized in this laboratory and demonstrated to be an inhibitor of S-adenosylhomocysteine hydrolase, was shown to have potent activity against monkey ebola virus.

Further developments and improvement in synthesis for the fluoro-dideoxynucleosides will potentially benefit the anti-HIV drug discovery and development program. Demonstration of the acid stability suggests that an oral formulation may be appropriate.

Oxetanocin, a naturally occurring nucleoside with a four-member sugar, has anti-HIV activity. Expansion of the unusual ring to five members was assocoated with retention of anti-HIV activity.

Work continues on the development of inhibitors of both protein kinase C and tyrosine kinase. The most likely conformation of diacylglycerol has been established for its binding to the regulatory site of the PKC. Of interest, a compound has been identified that inhibits the autophosphorylation of the tyrosine kinase without affecting exogenous substrate phosphorylation.

The computerized molecular modeling techniques are currently directed to addressing the following laboratory projects: (1) design of competitive inhibitors of PKC, (2) design of dideoxynucleosides based on active members of the series, and (3) quantitative structure-activity relationships on inhibitors of tyrosine kinase.

Other projects completed by investigators in the laboratory included: (1) establishment of bioequivalence of solutions and tablets of HMBA in a phase I/II trial, (2) dideoxyguanosine was studied as an anti-hepatitis drug in ducks, and (3) fourty-two polypeptides were designed and synthesized as potential inhibitors of protease (a key enzyme in HIV maturation).

E. Laboratory of Biochemical Pharmacology (LBP)

Over the past three years, this laboratory has made significant contributions to the development of anti-HIV agents. Over the past year, the cellular pharmacology of 2',3'-dideoxy-2'-fluoro arabinosyladenine (fddA) has been investigated in several cell lines. This agent is acid resistant, and thus is potentially amenable to oral administration. fddA is more resistant to deamination than ddA, and anabolism to the diphosphate and the triphosphate is more energetic in comparison to ddA.

Study of the cellular pharmacology of 2',3'-dideoxyguanosine (ddG) have been initiated over the past year. This agent has interest because of activity against hepadnaviruses, in addition to its

activity in inhiting the replication of HIV and other retroviruses. ddG enters cells by diffusion and is phosphorylated to ddGTP in concentrations adequate to inhibit retroviral reverse transcriptase. Inhibitors of IMP dehydrogenase increase the formation of the ddG nucleotides, and the anti-HIV activity is markedly increased by combinations like ddG and ribavirin.

The laboratory has continued its collaboration with the Clinical Oncology Program in completing work on the pharmacology of ddI, and plans continued collaboration with the initiation of clinical trials of CPE-C.

Finally, this laboratory has been incorporated into the Laboratory of Medicinal Chemistry. This administrative change was accomplished following the recommendation of the Board of Scientific Counselors, DCT, NCI.

Extramural Branches of the Developmental Therapeutics Program-

F. Drug Synthesis and Chemistry Branch (DS&CB)

The fundamental responsibility of this branch is the discovery of novel leads for drug development against cancer and AIDS. During the past year, approximately 7,000 new compounds were acquired from 677 suppliers (280 pure natural products; 6720 synthetic compounds). The DS&CB plays a key role in operation of the Acquisition Input Committee for both the cancer and AIDS screens. Approximately 200 compounds are tested weekly in the AIDS screen, and there are 300 compounds tested weekly in the cancer screen.

Active leads discovered in the AIDS screen over this period include: polyoxymetalates (NSC 622102); diaryl sulfones (NSC D624231); thiazolobenzimidazoles (NSC D625487), and a new oxathiin carboxanilide (NSC D629243).

Active leads are optimized through synthesis of prodrugs and congeners. Current efforts include synthesis of ellipticinium analogs, castanospermine analogs, diazo dyes, active ATA fractions, novel platinum analogs, and bombesin antagonists.

A total of 14 radiolabelled compounds were synthesized. Over 2,800 samples were shipped to other investigators for AIDS and cancer research.

G. Natural Products Branch (NPB)

This branch is responsible for the acquisition, isolation, structure determination, and testing of compounds from microbial, plant, and animal sources in the search for new leads in the treatment of cancer and AIDS. Contracts for the collection of plants from tropical rain forests and shallow-water marine organisms from the Indo-Pacific region have been in progress since September, 1986. A contract for the cultivation of cyanobacteria also began at that time. A contract for the cultivation of marine protozoa was awarded in March, 1989, and a project to cultivate unusual fungi was initiated at the Frederick Cancer Research and Development Center (FCRDC) in March, 1990. An initiative for the cultivation of marine anaerobic bacteria is planned for the near future.

Over 16,000 extracts of plant, marine, and microbial origin have been submitted for testing in the in vitro anti-HIV screen, and over 1,000 extracts have shown preliminary activity. Over 200 are being actively investigated with a view to isolation of the pure active agents. A new class of in vitro active agents, glycosulfolipids, has been isolated from cyanobacterial extracts. In addition, several new classes of active agents have been isolated from plant extracts. Over 5,500 extracts have been submitted for testing in the cancer screen, and results are currently being analyzed.

The natural product acquisition program is progressing very well. In the plant program, over 18,000 samples have been received of which over 8,200 have been extracted to yield over 16,400 extracts. The marine program has been progressing well in the collection of the shallow-water organisms with over 3,500 specimens having been received at a rate of 1,000 per year. Currently, more than 1,300 extracts have been prepared from the shallow-water specimens.

H. Biological Testing Branch (BTB)

The responsibilities of this branch include the development and implementation of the disease-oriented anticancer screen and the anti-HIV screen. Furthermore, the follow-up in vivo testing capabilities for both screens are the responsibility of this group. The BTB manages the large resource for the production, quality control, and the distribution of genetically and biologically defined rodents. These disease-free experimental animals are distributed to other NCI Divisions, NCI intramural investigators, other government agencies, and NIH grantee investigators on a

cost reimburement basis. The BTB maintains a large repository of experimental animal and human tumor lines for use by NCI. Tumors are also distributed to other cancer researchers on a cost reimbursement basis.

The current anticancer screen has reached an annual capacity of 15,000 tests per year with an expectation to reach the 20,000 annual capacity very soon. Prostate and breast cancer cell lines for screening are under development. Protocals for secondary in vitro confirmatory testing have been developed, and approximately 90% of the current in vitro panel lines are available for subsequent in vivo testing. The EORTC agreements are in place for in vivo studies for supplementing and confirming of results.

The AIDS in vitro screen is operational at a level exceeding 40,000 tests anually, with an expected annual test capability of 50,000 tests. Confirmatory testing of active agents include a syncitium assay and the production of HIV p24 antigen. Currently available in vivo models have limited application for the disease in man. Therefore, model develoment continues with several potential candidate models including a closely related lentivirus (BIV) and the SCID/NIH III mouse carrying HIV.

I. Information Technology Branch (ITB)

This branch supports the data processing, managing, and analysis needs for DTP's drug discovery and development programs in cancer and AIDS. The data for cancer drug testing extends from 1956 to the present, and includes all the results of the chemical and biological testing performed in DTP. In addition, the data for the AIDS screen was initiated in mid-1987.

Last year, the DTP computing systems underwent major revision in both hardware and database design. New and revised software have been developed over the past year, and additional changes will be required as the utilization of these systems has significantly increased.

A new cancer supplier report package was developed, and includes presentation of the actual dose-response data, an assessment of the sub-panel selectivity, and demonstration of the mean graphs depicting activities at the growth inhibition level, at the cytostatic and cytotoxic levels, respectively.

A triage system has been developed that permits separation of those compounds requiring closer inspection by the Screening Committee, and those

compounds that require no further evaluation because of non-selective, non-cytotoxic patterns. An on-line system which permits any user to access cancer supplier report forms by NSC number is operational.

An AIDS Decision Module was developed to document and track antiviral testing of compounds. Individual test results from various areas are brought together for agents, and a conclusion derived from the serial testing is provided. An on-line system has been developed for tracking the progress of agents within the system.

An entirely new system was developed for the presentation of in vivo data. Finally, a continuing effort is underway to make the DTP computer system more accessible for the average user. When completed, the menu system will list all of the available programs, and permit the user to select and execute their selection. On-line context-sensitive help will be available in place of an old instruction manual system.

J. Grants and Contracts Operation Branch (G&COB)

This branch is for the administrative and managerial focal point for the support and coordination of DTP extramural activities, including grants, contracts, and cooperative agreements.

Grants - The Biochemistry and Pharmacology Grant Program comprises all aspects of anticancer drug discovery and development research. At the end of fiscal year 1989, 332 grants exceeding $52 million were administered by the branch. During fiscal year 1990, a total of 385 grant applications were received. Approximately 25% are expected to be funded by the end of the fiscal year.

Although several important research findings were made over the past year, three grantees made outstanding contributions. Dr. Stephen Lippard's team at the Massachusetts Institute of Technology isolated cDNAs modified by the anticancer drug cisplatinum. These investigations may shed light on the mechanism of tumor cell repair of cisplatinum-induced DNA damage. Dr. Igor Roninson's team at the University of Illinois demonstrated a more sensitive method for measuring multidrug resistance utilizing the polymerase chain reaction, and subsequently demonstrated that human tumor cells have a non-uniform distribution of this property. Finally, Dr. Monroe Wall improved the synthesis for 9-amino-20(RS)-camptothecin, an agent selected for clinical development by the NCI based on encouraging preclinical antitumor data.

Contracts - The Developmental Therapeutics Program utilizes contracts to procure needed resources or services to enhance the efficiency of the drug discovery and developmental processes. This branch manages the entire contract process from conceptual development to the assessment of progress. Approximately $19 million in contracts were awarded over the fiscal year 1990.

Cooperative Agreements - The Developmental Therapeutics Program has used this mechanism since 1983 to implement the National Cooperative Drug Discovery Group Program (NCDDG). This important program represents a significant extramural investment in rational drug discovery and design. Recent developments in biomedical research are exploited for the potential development of novel therapeutic products.

The NCDDG Program has been successful in bringing five products to clinical trial including: (1) Hycamptamine, an analog of camptothecin with improved solubility; (2) HomoDES, a potent inhibitor of polyamine biosynthesis; (3) a monoclonal antibody called 225 IgG1, which binds with high affinity to the human EGF receptor and blocks EGF-induced activation of tyrosine kinase; (4) DAB486-IL-2, which is a novel diphtheria toxin-related interleukin-2 fusion protein; and (5) an anti-transferrin receptor antibody used to treat patients with hematologic malignancies.

K. Pharmacology Branch (PB)

This branch is involved with two basic aspects of preclinical drug development: (1) detailed therapeutic studies, and (2) pharmacokinetic studies, including method development on candidate agents for the treatment of either cancer or AIDS.

Contracts are utilized to explore in vivo therapeutic efficacy of new drugs in murine models with various routes of administration and treatment schedules. For cancer drugs, human xenograph and murine tumor models are used. In the development of agents for AIDS, murine retrovirual models (Rauscher leukemia R-MuLV and LP-BM5) are being used, but improved models are being developed that more closely approximate the human disease.

Detailed pharmacokinetic studies have been performed on a number of agents under development for the treatment of either cancer or AIDS: (1) Anticancer compounds include CPE-C, Penclomedine, Pyrazoloacridine, Camptothecin analogs, ellipticinium derivatives, a

cholera toxin, and some cytochalasins. Several combination studies included cisplatinum and aphidicolin glycinate; BCNU and O^6-methylguanine. Finally, a collaborative study with the Cancer Therapy Evaluation Program for the development of a tetraplatinum assay has been initiated.

Detailed drug studies for AIDS include: (1) polyoxometalate, (2) stibene aldehyde, (3) two derivatives of oxathiin carboxanilide, and (4) five additional discreet compounds and four ATA polymer fractions.

L. Toxicology Branch (TB)

This branch is responsible for the studies which focus on the hazards of new investigational agents to healthy organs in intact experimental animals. The end-organ toxicity is defined in addition to establishing the dose-responsiveness and schedule-dependency of the toxicity. Plasma drug concentrations are correlated to safety, toxicity, and in vitro efficacy. The reversibility of the toxicity is determined, and a safe starting dose for the phase I clinical trials in humans is established.

This year four new antineoplastics and eight anti-HIV agents were in phase IIA of preclinical toxicology. Three anticancer drugs were at stage IIB. Cyclodisone was dropped for excessive toxicity and formulation difficulties, and the other two (pyrazoloacridine and CPE-C) are entering clinical trial.

Drug combination studies were also performed for clinical toxicity information involving: MGBG, tegretol and DFMO, Carboplatin and BSO. An oral study with Ara-AC was also done.

Research projects in the Toxicology Branch over the past year have focused on innovative methods to compare metabolism between species to project both toxicologic and efficacy differences based on metabolism. A project has been launched to compare the metabolism of anti-HIV agents in lymphocytes from experimental species and humans. An attempt is also being made to compare in the AIDS screen, the antiviral activity of heat-inactivated serum samples taken from animals following in vivo treatment with the anti-HIV agent and an in vitro, direct exposure to the same drug. This type of investigation may permit an assessment of the antiviral activity of a specific agent in vivo.

M. Pharmaceutical Resources Branch (PRB)

The responsibilities of this branch are to supply high quality chemical substances and formulated products for investigative program use for the Division of Cancer Treatment and the AIDS clinical programs of the NIAID. This branch has four functional areas:

(1) Chemical Resources: The primary functions of chemical resources are to provide for resynthesis, large-scale production and procurement of chemical substances. This service supervises thirteen chemical preparation laboratories under contract with the NCI. The material must be produced under GMP conditions, and meet the highest product standards.

(2) Analytical: The analytical service resource provides for: (a) analytical characterization of new investigational agents, and (b) chemical analysis of formulated products. Currently, the analytical chemist supervises five analytical contracts that have the capability of characterizing a structurally diverse group of chemicals. The service monitors the protocals for analyzing chemical purity and stability, and is involved with the development of the appropriate analytical methods needed for the program.

(3) Pharmaceutical Research and Development: The dose form development service is responsible for conversion of bulk chemicals into pharmaceutical products suitable for clinical use in chemotherapy and AIDS programs. Approximately 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 tried, but newer techniques may be needed (emulsions, prodrugs, and complexation). The staff manage three pharmaceutical research and development contracts, and one combined research/development and pharmaceutical contract.

(4) Pharmaceutical Acquisition and Production: This service manages five pharmaceutical contracts with capabilities to produce a broad variety of pharmaceutical products. The service also manages a storage and distribution contract with computerized capabilities for accurate

accountability of the distribution of all investigational products, and a separate shelf-life contract.

During the past year, the service purchased drugs in excess of $2.5 million for the intramural patient population. Furthermore, the overall production of 700,000 injectable units and 400,000 oral dosage forms for general clinical distribution were managed by this service.

Over this reporting period, the staff of the PRB have managed and distributed an increasing number of varied biological products. The branch was responsible for manufacturing large quantities of bulk and formulated supplies of dideoxyinosine in response to a rapidly increasing demand for the drug while awaiting supplies from the clinical sponsor.

Research efforts have been directed at improvements in the methods for drug delivery. New parenteral formulations involving emulsions, microdispersion, and liposomal administration have been explored.

Table 1

COMPOUNDS THAT PASSED DECISION NETWORK (4/1/89 - 3/31/90)

NSC Number	Name	Compound Type*
	Decision Network IIA	
	Antineoplastic	
609974D	Discreet	S
155693D	Discreet	S
627505D	Discreet	S
603071	9-Amino-camptothecin	S
	Anti-HIV	
625487D	Discreet	S
624231D	Discreet	S
627708D	Discreet	S
624958D	Discreet	S
622102D	Discreet	S
623421D	Discreet	NP
007229	Suramin analog	S
618121D	Discreet	S
620753D	Discreet	S
619179D	Discreet	S
623310D	Discreet	NP
	Decision Network IIB	
	Antineoplastic	
614491	8-Chloro-cAMP sodium salt	S
338720	Penclomedine	S
366140	Pyrazoloacridine (4/24/89)	S
	Decision Network III	
	Antineoplastic	
366140	Pyrazoloacridine (2/5/90)	S
122758	*Trans*-retinoic acid	S
020256	Uridine	S
	Yttrium monoclonal antibody	B
363812	Tetraplatin	S
609699	Hycamptamine	S
345842	Mafosfamide cyclohexylamine salt	S

NSC Number	Name	Compound Type*
	Decision Network IV	
	Antineoplastic	
356894	Deoxyspergualin	SS
336628	Merbarone	S

* S = synthetic
NP = natural product
B = biologic
SS = semisynthetic (natural product modified synthetically)

Table 2

Working Groups of the Operating Committee

A. Cancer Drug Development

Cancer Drug	Chairperson
Cyclopentyl Cytosine	Dr. Tomaszewski
8-Chloro-cAMP	Dr. Zaharko
Camptothecins	Dr. Plowman
MX-2	Dr. Acton
Ellipticinium	Dr. Acton
Cytochalasin	Dr. Narayanan
609974D	Dr. Curt

B. Antiviral Drug Development

Antiviral Drug	Chairperson
624231D	Dr. Malspeis
625487D	Dr. Malspeis
627708D	Dr. Malspeis
629243D	Dr. Boyd

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CM 06191-03 OAD

ERIOD COVERED
October 1, 1989 through September 30, 1990

TLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Program Development Research Group (PDRG)

RINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI's (alphabetical listing):

M. Alley, Ph.D.	Pharmacologist	OAD, DTP, NCI
J. Beutler, Ph.D.	Expert	OAD, DTP, NCI
J. Blunt, Ph.D.	Visiting Scientist	OAD, DTP, NCI
M. Boyd, M.D., Ph.D.	Chief, PDRG	OAD, DTP, NCI
J. Cardellina, Ph.D.	Suprv. Research Chemist	OAD, DTP, NCI
K. Gustafson, Ph.D.	Sr. Staff Fellow	OAD, DTP, NCI
L. Malspeis, Ph.D.	Chemist	OAD, DTP, NCI

OOPERATING UNITS *(if any)*
Biological Testing Branch, DTP, FCRDC, NCI; Natural Products Branch, DTP, NCI; Program Resources, Inc., FCRDC; Office of the Special Assistant for Antiviral Evaluations, DTP, NCI

AB/BRANCH
Office of the Associate Director

ECTION

STITUTE AND LOCATION
NCI, Frederick Cancer Research and Development Center, Frederick, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
31	18	13

HECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

JMMARY 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 discovery research and development programs of the National Cancer Institute. To this end, PDRG investigators have made substantial progress in a number of interrelated areas, in cellular pharmacology, ultrastructure, immunochemistry, tissue culture, and tumor cell biology, and natural products chemistry. Highlighted in this year's report are studies aimed at the further refinement of the screening models and assay methodologies used in the NCI's high-flux, in vitro antitumor and anti-HIV primary drug screens. Also described are model-development and applications efforts aimed at the detailed follow-up evaluation of new lead compounds identified in the screens. Detailed biochemical characterizations of human tumor cell lines have included prostanoid biosynthesis, metabolic activation of 4-ipomeanol and molecular genetic approaches to the elucidation of the human lung cytochrome P450 hemoprotein monooxygenase system. New methodology has been defined for the establishment and propagation of certain "normal" (nontumor) lines in vitro. Extensive efforts have been made to develop an in vivo microencapsulation model for anti-HIV drug evaluations; however, results to date indicate this approach will not likely be feasible. Detailed studies of new lead prototype antitumor and anti-HIV compounds, both of synthetic and natural origin, have been underway; however, a more detailed report of these efforts is being deferred until the 1991 Annual Report (i.e., pending declassification of discreet structures).

Professional Personnel (cont'd)

T. McLemore, M.D., Ph.D.	Sr. Investigator	OAD, DTP, NCI
J. McMahon, Ph.D.	Biologist	OAD, DTP, NCI
R. Shoemaker, Ph.D.	Spc. Asst. R&D	OAD, DTP, NCI
P. Skehan, Ph.D.	Expert	OAD, DTP, NCI
S. Stinson, Ph.D.	Biologist	OAD, DTP, NCI
D. Vistica, Ph.D.	Pharmacologist	OAD, DTP, NCI

Others (alphabetical listing):

L. Decosterd	Guest Researcher	OAD, DTP, NCI
W. Lin	Visiting Fellow	OAD, DTP, NCI
M. Tischler	Visiting Fellow	OAD, DTP, NCI
J. Warren	IRTA Fellow	OAD, DTP, NCI
S-J. Yu	Visiting Fellow	OAD, DTP, NCI

New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. PDRG investigators have completed the development and evaluation of a rapid, sensitive, and inexpensive method for measuring the cellular protein content of adherent and suspension cultures in 96-well microtiter plates. The method is suitable for ordinary laboratory purposes and for very large-scale applications, such as the National Cancer Institute's disease-oriented in vitro anticancer drug discovery screen, which requires the use of several million culture wells per year. Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density in a computer-interfaced, 96-well microtiter plate fiberoptic reader. The SRB assay results were linear with cell number and values for both Lowry and Bradford assays of cellular protein, at densities ranging from sparse subconfluency to multilayered supraconfluency. The signal-to-noise ratio at 564 nm was approximately 1.5 with 1,000 cells per well. The sensitivity of the SRB assay compared favorably with sensitivities of several fluorescence assays and was superior to those of both the Lowry and Bradford assays and to those of 19 other visible dyes. The SRB assay provides a colorimetric end point that is nondestructive, indefinitely stable, and visible to the naked eye. It provides a sensitive measure of drug-induced cytotoxicity, is useful in quantitating clonogenicity, and is well suited to high-volume, automated drug screening. SRB fluoresces strongly with laser excitation at 488 nm and can be measured quantitatively at the single-cell level by static fluorescence cytometry.

Correlation of Screening Data Generated with a Tetrazolium Assay (MTT) versus a Protein Assay (SRB) Against a Broad Panel of Human Tumor Cell Lines. Large scale in vitro drug screening requires a very efficient automated assay. PDRG scientists have previously shown that tetrazolium-based assays (MTT) as well as a new protein assay procedure (SRB; see above) can be utilized. Recently, PDRG staff, in collaboration with DTP extramural staff and PRI scientists, have extensively compared the two assays in a drug screening format. In both

systems, the cell lines were incubated for 24 hrs followed by 48 hrs with the test compound. 243 compounds were tested at 5 dose levels (with $\log_{10}$ intervals) against up to 38 human tumor cell lines, from a broad spectrum of tumor types, with both MTT and SRB performed on the same day. In addition, 28 compounds were retested with MTT on a subsequent day, and 26 were retested with SRB on a subsequent day. Differences in cell survival percentage, between the two assays, are given below. 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, and the median correlations given.

	Cell Survival Differences				Correlations	
	≤ 10%	10-20%	20-40%	> 40%	Rho	K_c
MTT-SRB	66.6%	23.6%	8.6%	1.2%	.83	.82
MTT-MTT	67.8%	21.1%	8.0%	3.0%	.85	.84
SRB-SRB	75.4%	17.6%	5.4%	1.6%	.84	.82

These results indicated that under the experimental conditions employed and within the limits of the data analyses, the assays performed quite similarly; however, SRB has superior practical advantages for large-scale screening.

Dependence of MTT Reduction Upon Cellular NADH and NADPH. The hydrogen acceptor MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide] is commonly utilized to estimate cellular viability in drug screening protocols. Previous studies by PDRG investigators indicated that depletion of D-glucose from culture medium of tumor cells is accompanied by a reduction in MTT specific activity (MTT formazan formed/cell). In order to more fully define the relationship between MTT specific activity and culture medium composition, a series of additional experiments were undertaken by PDRG scientists using the K562 chronic myelogenous leukemia. The results indicated that substitution of D-galactose for D-glucose as the principal carbohydrate in growth medium resulted in a 2-4 fold reduction in MTT specific activity and a decrease in the cellular concentration of the reduced pyridine nucleotides NADH and NADPH, cofactors required for the reduction of MTT These results suggest that the specific activity of MTT is highly dependent upon maintenance of cellular concentrations of these reduced pyridine nucleotides.

Growth and Relative In Vitro Drug Sensitivity of Multiple Human Tumor Cell Lines. In the NCI's in vitro, anticancer drug screen, 60 cell lines are continuously exposed to chemical agents for a 48 hr period. The diversity of these cell lines is reflected in their growth, as measured by an increase in cell protein and their relative drug sensitivity (RS). The increase in protein ranges from 30-100%. PDRG and PRI scientists have collaboratively examined the relationships between relative growth rates and drug sensitivity of the panel lines. The most rapidly growing cell line panel is the colon cell line panel, followed by the leukemia/lymphoma (L/L) cell line panel. RS was measured by two parameters, IC_{50} (treated (T)/control (C) x 100 = 50%) and GI_{50} (T-Tz/C-Tz x 100 = 50%) where Tz is a measure of cell protein at the beginning of drug incubation. Comparing and ranking the IC_{50} values showed

the L/L panel as the most sensitive, followed by the colon panel. Growth rate and IC_{50} showed a positive relationship $r^2 = 0.67$) indicating that rapid cell growth is accompanied by greater drug sensitivity. When GI_{50} values were evaluated, the L/L panel still appeared as the most sensitive panel overall, whereas the colon panel appeared relatively resistant, and the relationship between RS and growth rate was lost ($r^2 = 0.21$). These results indicate that the T_2 calculation may allow more meaningful comparisons of relative cellular drug sensitivities among cell lines of widely diverse doubling times.

Selective Cytotoxicity of Pseudomonas Exotoxin Coupled to an Antibody Against an Ovarian Cancer Cell Line in 56 Human Tumor Cell Lines. Pseudomonas exotoxin (PE) coupled to a monoclonal antibody (OVB-3) raised against a human ovarian cancer cell line was tested for differential cytotoxic activity against 56 cell lines derived from human cancers of the breast, central nervous system, colon, lung, ovary, kidney and from melanomas, leukemias and lymphomas. In addition, reaction of OBV-3 with each of the cell lines was assessed immunocytochemically by PDRG investigators. A high degree of selective toxicity against mucinous adenocarcinoma cell lines of the ovary, colon, breast, and to a lesser extent lung, was demonstrated by PE-OVB-3. In general, there was a very good correlation between OBV-3 binding and cytotoxicity. It is concluded that coupling of PE to OBV-3 is an effective means for targeting the otherwise non-specific in vitro toxicity of this agent.

A 3-Dimensional Anticancer Colony Extinction Assay for Plastic-Attached Cells. Three-dimensional tumor architecture restricts drug, nutrient, and waste diffusion, creates a spatial mosaic of doubling times, and regulates cell function through intercellular communications. These attributes, critical to the success or failure of chemotherapy, are poorly modeled by subconfluent cultures. PDRG investigators have developed a simple method for constructing 3-dimensional colonies attached to plastic. Cells are placed in 10-25 μl drops in the center of wells of 6-, 12- or 24-well plates. Cell number depends upon growth characteristics, but is typically 1,000-2,000 cells per well in 12-well plates. After cells have attached, the wells are flooded with growth medium. The large excess of growth medium per cell allows colonies to grow for several weeks without feeding before decline phase sets in. This permits long-term recovery studies without feeding. Colonies are grown for a week before exposure to test compounds. During this time they grow to macroscopic size and are extensively multi-layered. Drug efficacy is measured as the regression or extinction of preformed colonies, whose chemosensitivity frequently differs from that of subconfluent cultures. Like multicellular spheroids, these 3-dimensional multilayered colonies appear to provide a superior in vitro model of solid tumor architecture.

Improved Efficiency of Soft-agar Culture Analyses Afforded by Specialized Microcomputer Software. Previous studies by PDRG investigators have demonstrated the suitability of the soft-agar culture (SAC) format for growth and experimental drug evaluations in a wide variety of human tumor cell lines In the current PDRG study, a microcomputer equipped with specialized software was interfaced with an image analysis system for the purpose of efficient data processing, a factor which previously has limited the utility of SAC assays in experimental drug evaluations. Results to date indicate that complete drug

sensitivity profiles, multiple indices of drug effect, and a report can be generated within 15 minutes following completion of image measurements. Such analyses permit immediate review of results as well as identification of cultures requiring and/or benefiting from closer scrutiny. In addition, data files are compatible with database incorporation for purposes of comparing SAC sensitivities among cell lines as well as for comparing SAC assay profiles with those of other assay formats. These findings indicate that the new analysis system will permit use of SAC assays for moderate-scale drug evaluations.

A Rapid In Vitro Method for the Evaluation of Potential Antitumor Drugs Requiring Metabolic Activation by Hepatic S9 Enzymes. Metabolic activation is a prerequisite for the antitumor activity of certain drugs such as cyclophosphamide. In vitro assays require systems for metabolic activation to reveal the toxicity of such compounds for tumor cells. Although a number of methods utilizing systems for the in vitro metabolic activation of drugs have been published, practical assays applicable to large scale screening for such agents have lacking. PDRG investigators found that incorporation of a liver subcellular fraction (S9) into an established cell growth inhibition assay (microculture tetrazolium assay) significantly increased the cytotoxicity of cyclophosphamide. Under optimal conditions, the 50% growth inhibitory concentration was decreased in the presence of S9 from more than 600 μg/ml to less than 4 μg/ml, depending upon the cell line. The method also proved suitable for studies of metabolic detoxification (enzymatically or non-enzymatically) by conjugation reactions. For example, glutathione (5mM) markedly reduced the cytotoxicity of activated cyclophosphamide. In contrast, the addition of UDP glucuronate (10 mM) in the presence of the UDP-glucuronosyltransferase activator UDP-N-acetylglucosamine (10 mM) had little effect on cyclophosphamide toxicity.

Primary Cultures of Proliferating Alveolar Type II Cells from Rat Lungs. PDRG investigators established culture conditions that supported the proliferation of primary cultures alveolar type II cells isolated from rat lungs. Proliferation was found to be dependent on several variables including the pH and the composition of the culture medium. Maximum proliferation occurred when the type II cells were maintained in sealed culture vessels in F12 medium supplemented with 10 mM, pH 7.0, HEPES buffer. Under conditions where cell division was minimal, there was a rapid loss of differentiated type II cell morphology, with the cells becoming flattened and showing an extremely attenuated cytoplasm. In contrast, in the culture conditions where proliferation was maximal, the cells retained their cuboidal epithelial morphology, and contained numerous cytoplasmic inclusion bodies, throughout the 10 to 14 days of observation. Other experiments confirmed that the proliferating cell population consisted of alveolar type II cells. These included microcinematography, tannic acid staining for intracellular inclusions, intermediate filament analysis, the determination of phagocytic activity, and electron microscopy. These studies established that proliferating primary cultures of rat alveolar type II cells could be obtained from rats of different strains, sexes and ages. Moreover, the culture conditions employed are simple, inexpensive, and should be adaptable to establishment of such "normal" (i.e., nontumor) cell cultures from other species.

Fatty Acid Cyclooxygenase Metabolism of Arachidonic Acid in Human Tumor Cells. As part of the detailed biochemical characterization of human tumor lines for potential use in the NCI drug discovery screen, PDRG scientists have studied the profiles of prostaglandin (PG) and thromboxane B_2 (TxB_2) biosynthesis from endogenous arachidonic acid in 55 established cell lines derived from human tumors of the lung, colon, kidney, prostate, ovary, and the central nervous system (CNS). Employing the mean levels of PGE_2, $PGF_{2\alpha}$, TxB_2, PGD_2, 9α,11ß-PGF_2, and 6-keto-$PGF_{1\alpha}$ in calcium ionophore A23187-stimulated cells as an index of PGH synthase activity, the distribution in cell lines with activity >2 pmol/10^6 cells was as follows: lung (14 of 28 cell lines), colon (2 of 10 cell lines), ovary (1 of 5 cell lines), prostate (0 of 2 cell lines), CNS (0 of 3 cell lines) and kidney (1 of 7 cell lines). PGE_2 was the predominant species synthesized in the majority of cell lines (15 cell lines) exhibiting PGH synthase activity. $PGF_{2\alpha}$ levels were either equal to or higher than PGE_2 levels in 3 cell lines. TxB_2 levels >2 pmol/10^6 cells was evident in two cell lines. Further evaluation of PGH synthase activity according to histological subclasses of lung tumors indicated that PGH synthase activity (>2 pmol/10^6 cells) with cell lines derived from small cell carcinomas being devoid of significant PGH synthase activity (0 of 9 cell lines). The histologic subclasses of cell lines derived from non-small cell carcinomas of the lung with PGH synthase activity >2 pmol/10^6 cells was as follows: squamous cell (0 of 2 cell lines), adenosquamous (1 of 2 cell lines), bronchioloaveloar cell (2 of 2 cell lines), adenocarcinoma (8 of 10 cell lines) and large cell undifferentiated (3 of 3 cell lines). These findings suggest that prostanoid biosynthesis may be characteristic of certain histologic classes of human tumors, particularly in non-small cell carcinomas of the lung.

Metabolic Activation of 4-Ipomeanol in Human Lung, Primary Pulmonary Carcinomas and Established Human Pulmonary Carcinoma Cell Lines. 4-Ipomeanol (IPO) is a pulmonary-specific toxin which is metabolically activated by a cytochrome P-450 (P-450) pathway in lung tissue. This agent has been recently committed to clinical testing in lung cancer (LC) patients under the auspices of the National Cancer Institute. In current PDRG studies, IPO metabolism (determined via measurement of [^{14}C] IPO covalent binding) was evaluated in a diverse sampling of 18 established human LC cell lines as well as in normal lung and primary lung carcinoma tissue obtained at the time of thoracotomy from 56 LC patients. IPO covalent binding in LC cell lines ranged from 248 to 1047 pmole [^{14}C] IPO bound/mg protein/30 min with a mean ± SE = 547 ± 62.2. IPO metabolism in normal lung tissue ranged from 12 to 2007 pmoles [^{14}C] IPO covalently bound/mg protein/30 min with a mean ± S.E. = 549 ± 60 and in LC tissue from 0 to 2566 with a mean ± S.E. = 547 ± 60 ($p > 0.3$). When individual patients were divided into smokers and current nonsmokers, no effects of cigarette smoking were observed for either lung or lung tumor tissue ($p > 0.1$ in all instances). When the extent of IPO covalent binding was evaluated with respect to the histological classification of individual LC cell lines, a wide range of IPO metabolic activation was observed as follows: adenocarcinomas = 441 ± 99 (N=6); a squamous cell carcinoma = 370 (N=1); bronchioloalveolar cell carcinomas = 602 ± 292 (N=2); large cell undifferentiated carcinomas = 666 ± 193 (N=2); small cell carcinomas = 690 ± 132 (N=5) and mixed cell carcinomas = 424 ± 120 (N=2). Similarly, when the extent of variation in IPO metabolism was evaluated in fresh LC tissue

according to histological cell type, a wide range in activation was observed as follows: adenocarcinomas = 520 ± 52 (N=15); squamous cell carcinomas = 553 ± 70 (N=24); large cell undifferentiated carcinomas = 471 ± 89 (N=8); small cell carcinomas = 512 ± 160 (N=4); bronchioloalveolar cell carcinomas = 216 ± (N=2); and other primary LC = 447 ± 216 (N=3). When IPO metabolism was simultaneously compared in normal lung and LC tissue from individual patients, no positive correlation was observed ($r=0.10$; $p>0.30$). In approximately 50% of the LC patients, IPO covalent binding in lung was similar to that in normal LC tissue from the same individual. In 25% of the patients, IPO metabolism was greater in normal lung tissue than in LC tissue, and in the remaining 25% of patients, IPO binding was higher in tumors than in normal lung, suggesting a dysregulation of IPO metabolism in LC. These results clearly demonstrate a wide interindividual range of IPO metabolism in both normal and LC cells and indicate that a wide diversity of human LC do possess the metabolic enzyme system(s) necessary for the bioactivation of IPO to a potentially cytotoxic intermediate. Therefore, the continued exploration of any possible therapeutic potential of IPO in LC patients appears warranted.

Altered Regulation of Different Cytochrome P450 Genes in Pulmonary Carcinomas. Cytochrome P450s (P450) are implicated in the metabolic activation of procarcinogens in cigarette smoke condensate. Extensive investigation of specific P450 genes which are expressed in human pulmonary tissues has not, however, been previously performed. PDRG investigators have studied the expression of 1A1, 2B1, 2D1, 2F1, and 4B1 genes in normal lung (NL) and primary lung cancers (LC) from 75 LC patients, including 28 current cigarette smokers (SM), 39 former smokers (FS) and 7 nonsmokers (NS). When 1A1 gene expression (GE) was investigated, 90% of NL from SM, but none of the NL from FS or NS expressed the gene. When LC were studied, only 47% of SM demonstrated 1A1 GE and LC tissue from FS or NS also exhibited 1A1 GE even in the absence of SM. Detectable 4B1 GE was observed in all NL studied, but when LC were studied, predominately only adenocarcinomas (64% expressed this gene, particularly the bronchioloalveolar cell subtype (100%), suggesting an association between this cell type and 4B1 GE. Furthermore, unlike NL, 4B1 GE was absent in LC from SM compared to tumors obtained from FS or NS. When 2B1 and 2F1 were evaluated, GE occurred in all NL studied irrespective of SM, but GE was absent in all LC tissues studied. Although an association between 2D1 activity and LC has previously been suggested, no 2D1 GE was noted in either NL or LC tissue. These results demonstrate altered regulation of 1A1, 2B1, 2F1 and 4B1 GE in individual LC compared with NL and support their potential role in pulmonary carcinogenesis, normal lung homeostasis and developmental therapeutics of LC.

CYP1A1 Expression in Lung Cancer Patients: Evidence for Cigarette Smoke-Induced Expression in Normal Lung and Altered Gene Regulation in Primary Pulmonary Carcinomas. The major polycyclic aromatic hydrocarbon (PAH) inducible-cytochrome P4501A1 gene (CYP1A1) is presumed to be important in pulmonary carcinogenesis and toxicology because its product, the cytochrome P4501A1 (CYP1A1)-dependent monooxygenase, transforms selected xenobiotics (including PAH procarcinogens in cigarette smoke) to potent carcinogenic metabolites. CYP1A1 expression has not, however, been previously demonstrated in human pulmonary tissue. Therefore, PDRG investigators have studied CYP1A1

expression in normal lung and primary pulmonary carcinoma tissue obtained at thoracotomy from 56 lung cancer patients. When Northern hybridization analyses were performed, 17 of 19 (89%) and 0 of 5 (0%) of normal lung tissues from active cigarette smokers and nonsmokers, respectively, expressed the normal 2.8 kb CYP1A1 mRNA. In addition, a time-dependent decrease in expression of CYP1A1 was noted in normal lung from individuals who were former smokers, with a decrease in expression occurring as early as 2 weeks following cessation of cigarette smoking and becoming undetectable in all patients who had stopped smoking >6 weeks prior to study. When CYP1A1 expression was evaluated in lung cancers, 1 of 4 (25%) tumors from nonsmokers; 2 of 24 (8%) tumors from former smokers; and 7 of 15 (47%) tumors from cigarette smokers had detectable mRNA levels. In addition, an approximately 10 Kb CYP1A1 RNA species (not detectable in normal lung tissue) was observed in 50% (5 of 10) of the lung cancer tissues which expressed CYP1A1. There was no association between CYP1A1 expression and lung cancer histologic cell type nor between matched normal lung and tumor tissue CYP1A1 mRNA levels obtained from individual lung cancer patients. These results demonstrate a positive association between active cigarette smoking and CYP1A1 expression in normal human lung tissue. Moreover, CYP1A1 expression was documented in many pulmonary carcinomas and altered regulation of the gene was also observed in several lung tumors.

Differential Expression of CYP4B1 in Normal Human Lung and Primary Pulmonary Carcinomas. PDRG staff have investigated the expression of mRNA coding for a human lung-specific P450 gene, designated CYP4B1, in surgically resected normal human lung and pulmonary carcinomas obtained at the time of thoracotomy from 26 primary pulmonary carcinoma patients and in 19 continuous human lung cancer cell lines. The patient population included 15 current smokers (SM) 8 former smokers (FS), who had not smoked for at least 2 months prior to study, and 3 nonsmokers (NS). When normal lung tissue was evaluated, the CYP4B1 mRNA was present in 100% (20/20) of the tissues examined irrespective of smoking history with approximately a 20-fold variation in levels among different individuals. When lung tumors were examined, only 44% (8 of 18) demonstrated detectable CYP4B1 expression. Interestingly, only one pulmonary carcinoma from a SM expressed detectable CYP4B1 mRNA levels, while 57% (4/7) lung tumors from FS and 100% (3/3) tumors from NS demonstrated expression of this gene. Furthermore, 85% (6/7) of the adenocarcinomas were positive for CYP4B1 expression and 100% of lung tumors from the subclass of adenocarcinomas representing bronchioloalveolar cell carcinoma expressed CYP4B1. In all cases, for those pulmonary carcinomas that expressed this gene, mRNA levels were only approximately 20% of those observed for the corresponding normal lung tissue from individual patients. Consistent with what was observed in fresh tumor tissue, only 1 lung cancer cell line, a bronchioloalveolar cell carcinoma, demonstrated detectable CYP4B1 gene expression. Thus: (I) constitutive CYP4B1 expression occurred in all normal lung tissue regardless of smoking status, (II) there was greatly decreased CYP4B1 expression in primary pulmonary carcinomas from SM compared to those from NS or FS, suggesting altered gene expression in tumors associated with SM, and (III) CYP4B1 expression occurred predominantly in pulmonary adenocarcinomas, particularly the bronchioloalveolar cell carcinoma subtype.

Anti-HIV Drug Activity In Vitro: Impact of Infection Methodology and Virus Infectivity. The National Cancer Institute's in vitro, anti-HIV drug screen depends on virus-induced cytolysis of T-lymphoblastoid, CEM-SS cells. Cytoprotection is estimated by metabolic reduction of the tetrazolium salt XTT to its soluble formazan by surviving cells. PDRG intramural staff, DTP extramural staff and PRI staff have continued their collaborative efforts to further develop and refine the screening model. In experiments performed during the past year, it was determined that antiviral activity was influenced by the quantity of virus input (MOI) and the status of virus replication at the time of drug addition. These factors were, in turn, influenced by infection methodology and the ratio of infectious to noninfectious arterial (infectivity ratio) in virus stocks. Use of virus stocks with low infectivity ratios, bulk infection of target cells or increasing MOI, temperature of infection or the interval between infection and drug exposure, decreased assay sensitivity, increasing effective drug concentrations (EC_{50}) required and reducing total antiviral protection. Employing virus stocks with high infectivity, infecting target cells directly in microculture wells, lowering MOI, reducing the temperature of infection and the interval between infection and drug exposure, decreased EC_{50} and improved overall antiviral protection. These data suggest that conclusions pertaining to in vitro drug-induced antiviral protection or drug resistance (e.g., as with AZT) should take into account factors which impact both the rate and status of virus replication.

A Semiautomated, Multiparameter Assay for Anti-HIV Drug Screening. PDRG investigators have developed a semiautomated, multiparameter assay which addresses a full range of criteria by which to characterize the activity of antiviral agents against T-lymphoblastoid cells acutely or chronically infected with HIV. Concurrent with a metabolic tetrazolium assay (XTT), cellular viability (BCECF), total DNA content (DAPI), supernatant reverse transcriptase activity, p24 core antigen production and the synthesis of infectious virions can be assessed from a single well of a 96-well microtiter plate. Several known HIV inhibitors, AZT, ddC, dextran sulfate and prostratin were used to establish and validate the assays. Assay modifications have been made to increase automation and more importantly to satisfy safety considerations when working with HIV and HIV-infected cells. The assays developed provide a safe, semiautomated, and reproducible multiparameter approach for screening compounds for antiviral activity. These assays yield a maximum amount of information with a minimum amount of sample and may detect active compounds that may be missed by single endpoint assays. In addition, this multiparameter assay may aid in the prioritization of potential anti-HIV compounds for clinical trials.

Interactive Laser Cytometric Analysis of Retroviral Protein Expression in HIV-infected Lymphocytic Cell Lines. PDRG investigators have developed an interactive laser cytometric method to examine the expression of HIV envelope glycoproteins gp160, gp41, gp120, and the core protein p24 in the HIV-infectable human lymphocyte cell lines H9, CEM-SS and C8166. This method allowed for the ultrasensitive detection of fluorescence signals at the single cell level and, when combined with specific anti-HIV antibodies, permitted unique quantitative detection of HIV antigens. Indirect immunofluorescence assays with monoclonal antibodies directed against gp120 revealed that a large

proportion of lymphocytic cells strongly expressed gp120-associated fluorescence consistent with $HTLVIII_{rf}$ infection. Certain monoclonal and polyclonal antibodies were also effective in quantifying gp160, gp41, and p24 expression. In addition, expression of these antigens was found to vary significantly within 48 hrs. Significant loss (≥50%) of gp120 expression was observed when cells were treated with 1.0μM AZT. The expression of the HIV-associated protein markers gp160, gp41, and p24 was detectable 24 hrs after infection of C8166, a cord blood lymphocytic cell line. C8166 cells expressed an additional 6 to 10-fold increase in gp120 in 48 hrs as well as a 3 to 4-fold increase in gp160, gp41, and p24. AZT (0.01 and 0.1 μM) decreased the expression of gp120, gp160, and p24 in a dose-dependent fashion. This new application of interactive laser cytometry permits early, sensitive, and statistically-based distinctions in the expression of HIV-associated antigens in infected target cells at the single-cell level, and allows detection of important changes in HIV-associated antigen expression and the kinetics thereof. This technology may thereby provide a powerful new tool for investigations of HIV biology as well as facilitate the discovery and characterization of novel antiviral agents.

Mechanistic Studies on Oxathiin Carboxanilide, A Novel Potent Inhibitor of HIV Reproduction. Oxathiin carboxanilide (NSC 615985) was found to be highly active in the NCI's AIDS antiviral screen. PDRG investigators have collaboratively studied the mechanism of action of this agent. The compound inhibited virus reproduction; infectious virus, and extracellular viral p24 antigen. Extracellular viral reverse transcriptase was inhibited at concentrations (0.5μM) of NSC 615985 far below those which produced cytotoxicity (> 100μM). NSC 615985 had no direct effect on virions of HIV, or on the enzymatic activities of HIV reverse transcriptase or HIV protease. The point of action of NSC 615985 within the virus reproductive cycle was different from that of active nucleosides (e.g., azidothymidine or dideoxycytidine); limited treatment of newly infected cells with NSC 615985 had little effect on the progress of infection, while late addition and continued treatment effectively prevented virus-induced effects. The compound, benzoic acid, 2-chloro-5-[5,6-dihydro-2-2-methyl-1,4-oxathiin-3-yl) carbonyl] amino-isopropyl ester, was originally synthesized as a potential fungicide.

Cellular Microencapsulation Technology for the Evaluation of Anti-HIV Drugs In Vivo. PDRG scientists have investigated the feasibility of microencapsulation technology for the evaluation of anti-HIV drugs in vivo. Central to the development of this in vivo assay was the ability to place human cells in microcapsules with semipermeable membranes for implantation into test animals. The anti-HIV assay would involve the microencapsulation of human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV; the encapsulated cells would then be implanted into athymic nude mice and recovered after drug treatment in vivo. A positive antiviral effect of the test substance would be indicated by growth or survival of the virus-infected cells contained in the microcapsules. Several HIV-sensitive cell lines of the T-lymphoid, monocytoid and non-lymphoid origins were examined for growth in microcapsules in vitro and in vivo. Light- and electron-microscopic analysis of the

capsules, and the human cells contained therein, revealed the invasion of mouse immune cells and other adverse effects, which could not be overcome by any of numerous technical modifications attempted. We conclude that the application of cellular microencapsulation technology to in vivo drug testing protocols, where immunogenic reactions occur such as those observed, is not feasible.

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ANNUAL REPORT OF THE LABORATORY OF MOLECULAR PHARMACOLOGY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

The major goal of the Laboratory of Molecular Pharmacology (LMP) is to obtain basic knowledge that could be applicable to the development of new drugs and strategies for the selective killing of human tumor cell types.

DNA Topoisomerases as Targets of Anticancer Drugs (Y. Pommier & K. W. Kohn)

DNA topoisomerases continue to receive increasing attention as targets for chemotherapy and are an ongoing focus of work in the LMP. Investigation of topoisomerases as anticancer drug targets grew out of early studies in the LMP using DNA filter elution methodology which we had developed. This work had shown that several clinically active anticancer drugs, including doxorubicin, amsacrine and some other DNA intercalators, affect DNA in a manner that was suggestive of an effect on a topoisomerase. Subsequent work in the LMP developed this hypothesis further and contributed to the identification of topoisomerase II as the molecular target. Our studies have also concentrated on etoposides (VP-16 and VM-26) as specific inhibitors of topoisomerase II and on camptothecin derivatives as specific inhibitors of topoisomerase I.

During the past 2 years, we have been increasingly concerned with the factors and mechanisms which govern how drug-induced DNA-topoisomerase lesions lead to cell death, and this area is in our view crucial to the origin of selective cytotoxicity and chemotherapeutic potential. A new finding during the past year is that calcium depletion can protect remarkably well against the cytolethal consequences of certain drug-induced DNA-topoisomerase lesions.

Since topoisomerase II is a major component of the nuclear matrix, we asked whether sites in DNA where the enzyme causes strand cleavage tend to be within matrix-association regions (MAR). In a comprehensive analysis of the SV40 genome, we found this to be the case particularly for DNA-topoisomerase cleavage lesions induced by doxorubicin. The anthracyclines thus may block DNA-topoisomerase II selectively at certain physiologically crucial locations.

We have investigated the base sequences at doxorubicin-induced DNA-topoisomerase II cleavage sites in the MAR region, as well as some other regions of SV40 where frequent cleavage sites occurred. A remarkable finding was that doxorubicin-induced cleavage sites invariably have an A as the first upstream base relative to at least one of the two breaks produced by an enzyme molecule in the DNA duplex, and that this is the converse of what happens when the enzyme is used without drug, in which case the presence of A at these same positions inhibits cleavage. On the basis of this observation, we have proposed a testable structural model. Anthracyclines are in this regard unique among topoisomerase inhibitors so far examined. We are investigating in similar fashion the base sequence requirements of other topoisomerase inhibitors in the hope of obtaining clues to the molecular

interactions that determine selectivity of action in the genome. We have, for example, noted an unusually strong reaction site induced by amsacrine at a c-myc promoter. We are also studying the determinants of selectivity in oligonucleotides synthesized according to observed hyperreactive sites. These selectivities may determine therapeutic potential, and our studies may suggest how drug structures might be modified to enhance these selectivities.

Mechanism of Action of Antitumor Alkylating Agents (K. W. Kohn)

Since nitrogen mustards such as cyclophosphamide and melphalan continue to play a major role in cancer chemotherapy, despite their nonspecific chemical reactivities, we are continuing to investigate the molecular mechanisms of action of these drugs. Even a modest increase in selectivity in alkylating agents designed on the basis of new mechanistic insights could have substantial clinical impact.

In collaboration with the Drug Synthesis and Chemistry Branch, we have obtained a homologous series of mono and bifunctional nitrogen mustards in which the mustard group is connected by way of a hydrocarbon chain of variable length to an acridine moiety. The acridine ring intercalates in DNA and positions the mustard group so that it alkylates selectively certain positions in DNA, depending upon base sequence. We are investigating the dependence of base sequence selectivity and cell killing on the length of the connecting chain and on the mono or bifunctionality of the alkylating group. Our findings suggest that the monofunctional compounds are unusually potent cell killers, compared with other monofunctional nitrogen mustards or simple intercalating compounds. Since monofunctional mustards cannot form covalent crosslinks, they constitute a mechanistically distinct class of drugs.

A major new finding is that the DNA sequence selectivity of intercalating mustards is affected in a systematic way by the length of the connecting chain. In particular, reduction of chain length to 2 or 3 restricts the reactivity almost entirely to GG sequences. Since runs of G's are common features of certain gene regulatory regions, including several oncogenes, and since existing therapeutic mustards have this same selectivity to a lesser degree, the new compounds might be therapeutically superior.

In another type of approach, we are using our DNA alkaline elution technique to determine the intracellular pharmacokinetics of DNA crosslinking. We have found significant differences among some of the common nitrogen mustards in the rates of formation and removal of DNA crosslinks. The intracellular pharmacokinetics accounted for most sensitivity differences that were found among several human tumor cell lines. These data might be combined with whole body pharmacokinetic data to generate comprehensive models which include, as compartments in the model, molecular lesions at the presumed intracellular targets.

Tubulin as a Site for Pharmacologic Attack (E. Hamel)

This is a new area of investigation in our Laboratory, brought about by the transfer Dr. Ernest Hamel. Tubulin, the major protein in microtubules of

the mitotic spindle and of the cytoskeleton, is the target of action of at least one important class of anticancer drugs: the vinca alkaloids. Taxol, a promising new agent, also attacks microtubule functions in tumor cells. This project investigates new types of tubulin binders derived from natural products, in order to define the biochemistry of the interactions, and where possible compares analogs from which structure-activity information can be obtained.

Combretastatin, derived from a South African tree and isolated by G.R.Pettit, was found to bind to tubulin at the colchicine site and to inhibit tubulin polymerization. Two combetastatin analogs of increased potency were identified and are the subject of biochemical investigation. The compounds differed from colchicine in the rate and temperature-dependence of tubulin binding; a new model for the colchicine site was proposed.

Dolostatin 10, the most active of a series of novel cytotoxic peptides isolated from a marine animal by G.R.Pettit, is being studied intensively. New findings are that it noncompetitively inhibits the binding of vinca alkaloids to tubilin and interferes with the tubulin binding and hydrolysis of GTP. Dolostatin stereoisomers and sub-peptides, synthesized by Dr. Petit, allowed structure-activity studies which provided new clues to the molecular details of the interaction. In addition, certain other cytotoxic dolostatins were noted to block mitotic microtubule function, but unlike other mitotic inhibitors, did not have a discernable effect on tubulin; the mechanism is being investigated.

A tubulin inhibitor now of clinical interest is taxol. A project has been initiated to study the taxol binding site in tubulin by means of photoaffinity compounds. The synthesis of a series of photoaffinity analogs of taxol is in progress.

In addition to the effects of novel inhibitors, microtubules are being investigated from the point of view of interactions between tubulin and associated proteins, as well as interactions involving nucleotides and divalent metal ions. Of particular note is that 2′3′-dideoxyguanosine nucleotides can replace GTP in tubulin polymerization, but the polymer is abnormally short and stable, resembling the abnormal tubulin polymers formed in the presence of taxol. An understanding of these interactions is likely to be essential to the full comprehension of the drug effects.

DNA Damage and Repair in Specific Regions of the Genome (V. Bohr)

The studies of preferential DNA repair in active genes, which have been carried out mainly using ultraviolet light and carcinogens, have increasingly focussed also on chemotherapeutic agents. Procedures were perfected to apply enzymatic and chemical methods to the quantitation of platinum and nitrogen mustard lesions in specific genomic regions.

There are several interesting new findings: (1) Nitrogen mustard adducts form to different extents in different genomic regions in CHO cells, and are repaired preferentially in the active dihydrofolate reductase (DHFR) gene. (2) In a comparison of two human ovarian cancer cell lines of different drug sensitivities, cisplatin adduct repair in the DHFR gene was similar in

the two lines, but DNA interstrand crosslink removal was slower in the more sensitive line. (3) A region upstream of the c-myc gene was found to be repaired (UV damage) more efficiently in B lymphoblasts from a plasmacytoma-resistant strain of mouse than in the same cell type from a plasmacytoma-sensitive strain; the DHFR gene in the two strains was repaired equally well. (4) Gene specific repair of UV damage in CHO cells was inhibited by a combination of camptothecin (a specific topoisomerase I inhibitor) and merbarone (reported to inhibit topoisomerases without induction of cleavage complexes) while there was no significant inhibition by either of these drugs alone.

Studies are continuing on gene specific DNA repair in various human DNA repair deficiency syndromes, and these studies could provide clues both to the mechanisms of origin of tumors and to possible mechanisms for selective cell killing by DNA-damaging drugs. In addition, we are investigating a possible role of gene-specific repair in multi-drug resistance, a possibility that has not previously been explored.

Regulation of Histone Synthesis (W. M. Bonner & C. L. Hatch)

Cell proliferation requires that histone and DNA syntheses be closely coordinated; if coordination fails, the cell is likely to die. If this coordination could be uncoupled in some types of neoplastic cells, tumors made up of those cells could become susceptible to some chemotherapeutic strategies, for example with antimetabolites. One part of this project aims to elucidate the metabolic processes and gene regulatory events that control the synthesis of different types of histones.

A likely key to histone regulation is the unique stem-loop structure present near the 3′ ends of most histone mRNA's, which may regulate degradation of the mRNA by a specific nuclease. We are developing assays for this specific nuclease. Oligonucleotides that mimic the stem-loop sequences will be used as possible tools for the manipulation of histone control in cells. Such oligonucleotides, if found to successfully uncouple histone and DNA synthesis, could be therapeutically useful. The activity of the nuclease is thought to be regulated by the amount of soluble histone (histone not in chromatin) in the cell. Sensitive methods developed in this laboratory were used to measure soluble histone pools. We have found that soluble histones seem to be complexed in negatively changed complexes; studies in progress seek to characterize these complexes.

Studies have also focussed on the H2A family of histone proteins; this core histone family is unique in that, as we discovered, its members contain separately conserved protein motifs. Two of the members, H2A.Z and H2A.X, which were originally discovered in this Laboratory, are also interesting because they are regulated differently from the major H2A variants. While synthesis of the major H2A proteins is synchronized with DNA synthesis during the cell cycle, the synthesis of H2A.Z and H2A.X occurs throughout the cell cycle. We have isolated and completed the sequencing of the H2A.Z gene and are presently sequencing the H2A.X gene. The H2A.Z gene was found to contain 4 introns, a GC-rich promoter region and 2 Alu repeat sequences. Preliminary data on the H2A.X gene indicates that, like most histones, it lacks introns.

H2A.Z mRNA was found to differ from mRNA's of most of the major histones in that it lacks a 3' stem-loop structure and has a poly-A tail, features consistent with H2A.Z protein synthesis in the absence of DNA synthesis. H2A.X protein synthesis also occurs in the absence of DNA synthesis, but in an unexpected and surprising result, we found that the H2A.X cDNA did contain the stem-loop structure followed by a downstream polyA tail. Proliferating cells were found to contain two types of H2A.X mRNA, one with the 3' stem-loop and another with the polyA tail. The ratio of the two mRNA forms was found to vary greatly among cell types, with tumor cell lines containing much more of the replication-linked form and a normal cell line containing much more of the replication-independent form. These results indicate that the regulation of histone synthesis is considerably more complicated than originally expected and are currently being pursued with great interest.

Studies of the H2A.Z promoter region with CAT assays have delineated the core promoter to about 230 base pairs upstream from the CAP site. Current studies are utilizing DNase I hypersensitive sites and gel retardation assays to identify cis-acting elements and their interacting factors. Just further upstream is a region which shows properties of a silencer in most of the cell lines we have tested. Interestingly this sequence seems to activate transcription in human embryonic carcinoma cells that are proliferating, but not in ones that are differentiated with retinoic acid. The interactions between the core promoter and this upstream region are being further characterized.

In related studies, we have found that the HIV TAT gene product interacts with the H2A.Z promoter leading to a downregulation of the gene. This is one of very few genes reported to be downregulated by TAT. Experiments to explore the physiological consequences of this effect are in progress.

Molecular Biology of Cell Injury (A. J. Fornace, Jr.)

This is a new project in the LMP, transferred with Dr. A.J.Fornace from the Radiation Oncology Branch. The work centers on the isolation and characterization of genes whose expression is induced by cell injury by agents such as ultraviolet light, heat and alkylating agents. It aims to determine how DNA damage inducible genes affect cell survival, particularly in cells treated with DNA damaging chemotherapeutic agent.

Dr. Fornace has isolated and sequenced some 20 different cDNA transcripts that are induced by DNA damage. Several of these are being investigated in further detail. One is a DNA single-strand binding protein and several exhibited abnormal expression in human DNA repair deficient cells.

Several of the transcripts were also associated with growth arrest, and genes of this class were given the designation gadd ("growth arrest and DNA damage inducible"). Such genes are of special interest because cell survival following DNA damage is often improved by transient growth arrest. A particularly interesting finding was that promoters from two different gadd genes contain areas of sequence homology, Since these areas did not correspond to any known transcription factor recognition sequences, they may contain new recognition sites for transcription factors that may specifically control these types of genes. The DNA damage responsive elements

in the two genes were localized through the use of CAT constructs. One of the genes was interesting also because it was strongly induced by x rays in human cells, and this induction was found to be deficient in cells from patients with the ataxia telangiectasia, a genetic disease characterized by radiosensitivity and increased cancer risk.

Dr. Fornace had also isolated the largest collection of mammalian cDNA clones for heat-shock genes. Some of these genes were found to be induced by DNA damaging agents. In particular, one of the genes, identified as the gene for ubiquitin, was found to be induced by alkylating agents, and another gene in the collection was induced by BCNU. A third gene, in an expression vector, increased the resistance of host cells to UV.

Also being studied is the expression DNA damage inducible genes and DNA repair genes in drug sensitive or resistant tumor cell lines. Early results showed increased expression of one of the gadd genes and of the gene for DNA polymerase beta (putative DNA repair polymerase) in a nitrogen mustard resistant line of Burkitt's lymphoma cells. The gene for the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase, was cloned, and was found to be underexpressed by a factor of 100 in mer$^-$ human tumor cell lines which have increased sensitivity to nitrosoureas. Measured underexpression of the alkyltransferase could serve to identify clinical tumors that would be responsive to nitrosoureas or related drugs.

Studies are planned to follow up on a recent report that an HIV promoter is strongly induced by DNA damage, mediated mainly by a protein kinase C activated transcription factor. The activation of this HIV gene will be studied following genotoxic and other types of stress, and the role of the gadd genes will be investigated. The aim is to gain insight into the effects of stress, particularly genotoxic stress, in possibly activating HIV genes and stimulating lytic viral infection.

Dr. Fornace had also isolated the largest collection of mammalian cDNA clones for heat-shock genes. Some of these genes were found to be induced by DNA damaging agents. In particular, one of the genes, identified as the gene for ubiquitin, was found to be induced by alkylating agents, and another gene in the collection was induced by BCNU. A third gene, in an expression vector, increased the resistance of host cells to UV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06140-14 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Regulation of Histone Synthesis

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
	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:
1.5	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.)*

One major objective is to elucidate how histone synthesis is regulated and how histones interact with DNA to form chromatin, not only during the presence of DNA replication in S phase cells but also during the absence of DNA replication in G1 and GO (quiescent) cells.

We have isolated some H2A cDNA clones which suggest that various specific mechanisms permit histone synthesis in quiescent cells to be from the same genes as used in proliferating cells. We are in the process of characterizing these mechanisms.

We have also 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. Current biochemical characterization of soluble histone suggests that it is bound in a negatively charged complex that sediments at 8-9s.

Isolating and characterizing the components involved in the complex but rigorous coordination of histone and DNA synthesis may lead to techniques or compounds of therapeutic value which disrupt this coordination in such a manner as to be selectively lethal to proliferating cells.

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.

Quiescent cells also synthesize histone at about 10% the rate found in proliferating cells, but it remains unclear how histone mRNA molecules found in quiescent cells differ from those found in proliferating cells.

Objectives:

1) To biochemically characterize the cellular components that may be involved in the mechanisms regulating histone mRNA stability relative to DNA replication.

2) To prepare synthetic oligomers that may mimic the 3′ stem-loop of histone mRNA. To use these oligomers to disrupt the replication-linked regulation of these mRNAs in order to elucidate the regulatory mechanisms and to test the possibility of using them as therapeutic compounds.

3) To characterize histone mRNAs from quiescent cells.

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

(5) Recombinant DNA technology.

Major Findings and Accomplishments:

1. To characterize components regulating histone mRNA stability.

Little is known about the specific mechanism that regulates the amount of histone mRNA relative to the rate of DNA synthesis. One component thought to be important is the soluble histone, that histone not bound in chromatin. 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 distinguishing 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. 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. 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.

A second compoonent important to the regulation of histone mRNA stability is a presumably specific nuclease which degrades the histone mRNA from the 3′ end. We are in the process of setting up an assay for this enzyme. The purpose of investigating this nuclease is that it may recognize the stem-loop on the 3′ end of the histone mRNA. Oligonucleotides that interfere with this recognition may lead to the uncoupling of histone synthesis from DNA replication, a situation that one may be able to exploit therapeutically.

2. Characterize histone mRNAs from quiescent cells

While replication-linked histone mRNA contains a 3′ stem-loop structure, histone mRNAs from quiescent cells are in many cases the same or very similar size. We have isolated some histone cDNAs that encode longer transcripts but which include the stem-loop motif. We are studying the involvement of these mRNAs in quiescent cells.

Significance to Biomedical Research and the Program of the Institute

Many compounds of therapeutic value in cancer interfere with DNA replication. The uncoupling of histone protein synthesis from DNA replication would also interfere with DNA replication.

Proposed Course:

1. To characterize soluble histone and study its interaction with other components involved in histone mRNA stability.

2. To assay for the histone mRNA nuclease and characterize.

3. To inhibit the nuclease action on histone mRNA in vitro and in vivio with oligonucleotides that mimic the 3′ histone mRNA stem-loop.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06150-09 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

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
	Richard Bertrand	Visiting Fellow	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)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	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.)*

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. We have found that the topoisomerase I-DNA complexes induced by camptothecin probably kill rapidly proliferative cells by interacting with DNA replication complexes. We have also determined that topoisomerase-mediated DNA breaks are not toxic in cells which have been depleted of calcium. 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 both increased P-glycoprotein and modified topoisomerase II. In addition, we have found that cells selected for resistance to vincristine have increased P-glycoprotein without drug-resistant topoisomerase II. Finally, we found evidence that two forms of DNA topoisomerase I are regulated by signal transduction pathways including protein kinase C.

PHS 6040 (Rev. 1/84)

GPO 914-918

1. Study the cellular events leading to cell death upon induction of topoisomerase-linked DNA breaks by drugs.

2. Determine the involvement of topoisomerase II in the pleiotropic resistance of cells selected by their resistance to anticancer drugs.

3. Isolate topoisomerases from resistant cells and study their regulation by protein kinases.

4. Determine the genomic localization of topoisomerase-induced DNA breaks.

Methods:

1. Standard cell culture and clonogenic assays.

2. Alkaline elution to quantify DNA breaks and DNA-protein crosslinks and to isolate topoisomerase-linked DNA fragments.

3. DNA hybridization with ^{32}P-labeled probes.

4. Preparation of nuclear extracts; topoisomerase purification by anion exchange chromatography; and immunoblotting with topoisomerase antibodies.

5. DNA topoisomerase assays with purified enzymes and DNA: filter binding, agarose gel or DNA sequencing gel electrophoresis and autoradiography of ^{32}P-end labeled DNA fragments.

Major Findings:

1. Cytotoxic Mechanism(s) of Topoisomerase Inhibitors in Chinese Hamster DC3F cells:

Although the stabilization of topoisomerase II cleavage complexes by etoposide (VP-16) has been recognized to be important for cell killing, the mechanisms leading to cytotoxicity remain to be elucidated. In an attempt to characterize the biochemical requirements for cell death, we examined the effect of Ca^{++} depletion on drug-induced cytotoxicity and DNA single-strand breaks (SSB) in chinese hamster DC3F cells. Four hour preincubation in a Ca^{++}-free medium or in complete medium containing 5mM EGTA protected against the cytotoxicity of VP-16. Under these conditions, DNA SSB induced by VP-16 remained similar to those of control cells. Furthermore cell-cycle analysis and thymidine pulse incorporation indicated that DNA synthesis and cell cycle distribution were similar in calcium-depleted and control cells. Drug cytotoxicity was restored progressively when cells were fed with complete medium or by adding calcium to the Ca_{++} -free medium for an additional 4 to 8 hours. Calcium depletion also protected against the cytotoxicity of camptothecin, hyperthermia and to lesser extent of nitrogen mustard and gamma radiation in DC3F cells. Similar results were obtained in human colon carcinoma HT29 cells. Our results suggest that Ca^{++}-dependent cellular processes are important in controlling the cytotoxicity of various potentially lethal DNA lesions.

2. 10,11-methylenedioxy-20-(RS)-camptothecin, a Topoisomerase I Inhibitor of Increased Potency: DNA Damage and Correlation to Cytotoxicity in Human Colon (HT-29) Cells:

We had previously shown that 10,11-methylenedioxy-20-(RS)-camptothecin (MDO-CPT) is a more potent inhibitor of purified DNA topoisomerase I than 20-(S)-camptothecin (CPT) (Jaxel et al., Cancer Res., 1989, 49: 1465-1469). The current studies compared the cytotoxicity and DNA damage induced by MDO-CPT and CPT in the human colon carcinoma cell line, HT-29. MDO-CPT was 7- to 10-fold more potent than CPT both for cytotoxicity (ID_{50}= 25 vs 180 nM) and production of DNA single-strand breaks (SSB). Kinetics of SSB formation and reversal were similar for MDO-CPT and CPT. DNA-protein crosslinks (DPC) were also produced by both drugs with a SSB/DPC ratio of 1/1. Moreover, no SSB were detected under non-deproteinizing conditions, indicating that both CPT and MDO-CPT produced protein-linked DNA single-strand breaks. A good correlation between cytotoxic potency and protein-linked DNA single-strand break production was observed for CPT and MDO-CPT, implying a causal relationship between drug-induced cytotoxicity and topoisomerase I inhibition. The cytotoxic potency of CPT in HT-29 cells was 1.8-fold less than in the rapidly growing Chinese hamster lung fibroblast (DC3F) cell line. In marked contrast, HT-29 cells were 25-fold more resistant to the topoisomerase II inhibitor, etoposide (VP-16) than DC3F cells. These findings suggest that human colon cancer cells may be more sensitive to camptothecins than to current chemotherapeutic drugs, including topoisomerase II inhibitors.

3. Association between Topoisomerase II Modifications and Increased P-glycoprotein in Multidrug Resistance Cell Lines:

We had previously reported that both multidrug resistant breast cancer cells (MCF 7/ADR) (Sinha et al., Cancer Res., 1988, 48: 5096-5100) and mouse leukemia L1210 cells (L1210/ADR) (Ganapathi et al., Cancer Commun., 1989, 4: 217-224) exhibit i) P-glycoprotein phenotype and ii) reduced drug sensitivity of nuclear topoisomerase II. We have now extended our study to P388 cell lines made resistant to different drugs (vincristine (VCR), adriamycin (ADR), mitoxantrone (MTT)) in order to test whether the association between P-glycoprotein increase and reduction of topoisomerase II sensitivity to drugs occurred similarly in all four cell lines. This was not the case since P388/VCR had increased P-glycoprotein without topoisomerase II modification, and both P388/ADR and P388/MTT had marked reduction of topoisomerase II-induced DNA cleavage associated with increase of P-glycoprotein.

4. Protein Kinase C Activity Influences Camptothecin-mediated DNA Strand Breaks and Cytotoxicity in DC3F/9-OHE, Chinese Hamster Cells Resistant to Topoisomerase II Inhibitors:

The purification of topoisomerase I from DC3F/9OHE revealed a copurification of kinase activity. By immunoblot, total nuclear lysate of DC3F/9OHE as compared to the parental line, DC3F, was markedly enriched with protein kinase C. We further examined the relationship between the kinase and topoisomerase I activities in DC3F/9OHE. Within 30 minutes after addition of 0.1 uM TPA (to stimulate protein kinase C), the DNA single strand breaks (SSBs), protein associated breaks (PASBs), and cytotoxicity mediated by camptothecin (CPT) were

enhanced by a factor of 1.5-2. When 0.1 uM TPA was added 24 hours prior to CPT, the levels of SSBs and cytotoxicity were 50% reduced. Also, pretreatment of DC3F/9OHE with 0.01 uM Bryostatin (to inhibit protein kinase C) for 3-5 hours prior to CPT, reduced CPT-mediated SSBs by 50%. These results are in agreement with our previous results with purified topoisomerases I (Pommier et al., J. Biol. Chem., 1989, 265: 9418-9412) and implicate protein kinase C as a regulator of topoisomerase I activity in this cell line.

Proposed Course:

1. Develop and study the cellular effects of new camptothecin analogs which could be developed as anticancer agents.

2. Identify the cellular lesions responsible for cell death after exposure to topoisomerase inhibitors.

3. Determine whether topoisomerase II alterations generally contribute to drug resistance in adriamycin-resistant cell lines exhibiting the pleiotropic phenotype.

4. Investigate the role of protein kinases and signal transduction pathways in the cytotoxicity of topoisomerase inhibitors.

Publications:

Covey JM, Jaxel C, Kohn KW, Pommier Y. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I, Cancer Res 1989;49: 5016-22.

Holm C, Covey J, Kerrigan D, Pommier Y. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I & II inhibitors in chinese hamster DC3F cells, Cancer Res 1989;49:6365-68.

Ganapathi R, Grabowski D, Ford J, Heiss C, Kerrigan D, Pommier Y. Progressive resistance to doxorubicin in mouse leukemia L1210 cells with multidrug resistance phenotype: reductions in drug-induced topoisomerase II-mediated DNA cleavage, Cancer Commun 1989;1:217-24.

Bhatia K, Pommier Y, Giri C, Fornace AJ, Imaizumi M, Breitman TR, Cherney BW, Smulson ME. Expression of the poly(ADP-ribose) polymerase gene following DNA strand breakage and effect of hyperexpression on DNA repair, Carcinogenesis 1990;11:123-28.

Pommier, Y., Kerrigan, D., Hartman, K. D., and Glazer, R. I.: Phosphorylation of mammalian DNA topoisomerase I and activation by protein kinase C, J Biol Chem 1990;16:9418-22.

Sinha BK, Politi PM, Kerrigan D, Pommier Y. Structure-activity relationship, cytotoxicity and topoisomerase II-dependent DNA cleavage induced by pendulum ring analogs of VP-16-213, Eur J Cancer, in press.

Holm C, Covey JM, Kerrigan D, Kohn KW, Pommier Y. Protection by DNA synthesis inhibition against cell killing by topoisomerase blocking drugs. In: Potmesil M, Kohn KW, eds. DNA Topoisomerases in cancer. New York: Oxford Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06161-07 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

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
	Ann Orr	Microbiologist	LMPH NCI
	Kurt W. Kohn	Lab Chief	LMPH NCI

COOPERATING UNITS *(if any)*

University of Texas Health Science Center, Dallas, TX 75235 (Dr. William T. Garrard), Rhone-Poulene Sante, Ivry sur Seine, France (Dr. J. F. Riou)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.6	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.)*

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.

DNA sequence analysis of cleavage sites induced by different inhibitors were investigated in order to study the molecular interactions between drugs and topoisomerase-DNA complexes. For some drugs (anthracyclines and camptothecins), a model emerged in which the drug seems to bind inside a cavity formed by the base pairs flanking the cleavage site (-1 and +1 bases) and the enzyme.

Drug effects in chromatin were 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 anthracycline-induced cleavage sites. Secondly, we found that topoisomerase II-mediated DNA cleavage was suppressed in reconstituted nucleosomes, and therefore drug activity depends upon chromatin structure. Finally, a major m-AMSA-induced DNA cleavage site was found in the promoter region of the c-myc gene, suggesting a role of topoisomerase II in transcription regulation and that the induction of cleavage sites in gene promoter regions may be important for the antitumor activity of topoisomerase II inhibitors.

Objectives:

1. Use purified enzymes and DNA fragments to compare drug potency and sequence selectivity and to select the most active topoisomerase inhibitors that could be studied in cells and animal tumor models.

2. Sequence topoisomerase cleavage sites in SV40 DNA and mammalian genes in order to map 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 DNA and in chromatin.

Methods:

1. Purification of DNA topoisomerases from mammalian cells in culture by anion exchange chromatography.

2. ^{32}P-end labeling of DNA fragments and oligonucleotides.

3.DNA sequencing gels and autoradiography to determine the DNA sequence at topoisomerase-induced DNA cleavage sites.

Major Findings:

1. Topoisomerase II Cleavage Sites in SV40 DNA; Relationship with Nuclear Matrix Attachment Sites:

Cleavage of ^{32}P-end-labeled DNA fragments was visualized by autoradiography of agarose and polyacrylamide gels. In the absence of drug, topoisomerase II cleavage sites were concentrated in a region between nucleotide 4100 and 4300. Anthracyclines also induced cleavage prominently in this same region, while m-AMSA and VM-26 induced cleavage with less selectivity. Binding of nuclear matrix from mouse cells was determined by a competition assay (Cockerill and Garrard, Cell 1986,44:273) and was found to be limited to the same DNA region that yielded the most intense cleavage site of DNA cleavage induced by topoisomerase II in the absence of drug and in the presence of anthracyclines. These results are in agreement with other findings demonstrating that topoisomerase II is a major component of the nuclear matrix and suggest that the strong antitumor activity of anthracyclines may be related to their selective action at nuclear matrix attachment sites.

2. DNA Sequence at Sites of Topoisomerase II-Induced DNA Cleavage:

We have located the topoisomerase II (Topo II) cleavage sites induced by anthracyclines in several strongly attacked regions of the SV40 genome, including the nuclear matrix associated region. Strong enzyme sites were always suppressed by doxorubicin. Of 97 doxorubicin sites, all had either an A at position -1 or a T at position +5. On the contrary, 90 sites of cleavage observed without drug had almost always a pyrimidine at position -1 and a purine at position +5. These

DNA sequence requirements are coherent with the observations that Topo II mainly induces DNA double-strand breaks with a 5′ overhang of 4 base-pairs. Results were similar with 4-demethoxydaunorubicin, 5-iminodaunorubicin. Thus, anthracycline-induced cleavage sites require an A at the 3′terminus of at least one of the break sites of a DNA double-strand break. This observation suggests that this A may be directly involved in a ternary complex between a doxorubicin molecule, the DNA and topoisomerase II.

3. DNA Sequence Requirements for Topoisomerase I Inhibition by Camptothecins:

We have shown previously (Jaxel et al., Nucleic Acids Res., 1988, 16: 11157-70) that camptothecin induces a major cleavage site at position 4955 of SV40 DNA. Also, DNA sequence analysis had revealed that cleavage sites had invariably a T at position -1 and that camptothecin enhanced cleavage preferentially at sites having a G at position +1. This observation led us to postulate an interaction between camptothecin and the +1 base. We have now studied enzyme-induced DNA cleavage in oligonucleotides corresponding to the strongest camptothecin site of SV40 DNA. Cleavage was induced in the 30-mer duplex oligonucleotide at the same site as in the native DNA sequence, but was less in the 20-mer, and was absent in the 10-mer. By substituting the +1 base, we found that in the absence of drug, cleavage was independent of that base and that camptothecin-induced DNA cleavage was greatest with a G, less with a C or an A, and was not detectable with a T. These results strongly suggest that the +1 base interacts with camptothecin, and that enzyme inhibition results from the formation of a ternary complex involving the bases flanking the cleavage site, the enzyme and camptothecin.

4. Nucleosomes Suppress Topoisomerase II-mediated DNA Cleavage Induced by Antitumor Topoisomerase II Inhibitors:

The effect of core histones upon topoisomerase II DNA was investigated by comparing the cleavage sites induced by four antitumor topoisomerase II inhibitors (teniposide, amsacrine, 5-iminodaunorubicin, 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. 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 in nucleosomes and other cleavage sites appeared stronger in DNA linkers. This is the first demonstration that nucleosome formation is critical for topoisomerase II activity and drug effects in chromatin.

5. DNA Cleavage Sites Induced by DNA Topoisomerase II Inhibitors in the Human c-myc Gene:

The c-myc gene has been shown to be approximately 20-fold more sensitive to amsacrine-induced DNA cleavage than the rest of the human cell genome (Riou et

al., Biochemistry, 1989, 28: 9104). The cleavage sites induced by purified Mouse leukemia (L1210) topoisomerase II were mapped in the absence and presence of amsacrine in a plasmid containing the 5′-flank of the Human c-myc gene (exon 1 and its flanking introns). We found a major enzyme cleavage site induced by amsacrine in the P2 promoter of exon 1 at position +172. Cleavage was on both strands with a 4 base pair stagger. This cleavage site was also detected in the absence of drug, and in cells treated with amsacrine, suggesting that topoisomerase II binds to the P2 promoter of exon 1 in chromatin. Less strong cleavage also occurred inside the P1 promoter and in the vicinity of the TATAA sequences of each of the two promoters of exon 1. Another region of strong cleavage sites was also induced by amsacrine approximately 200 base pairs upstream from exon 1. These results indicate that DNA topoisomerase II binds to the promoter regions of exon 1 of the c-myc gene and suggest that amsacrine-induced DNA breaks at these sites may be critical for antitumor 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 various DNA fragments, 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:

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 1989;264: 11354-59.

Pommier Y, Cockerill PN, Kohn KW, Garrard WT. Identification within the simian virus 40 genome of a chromosomal loop attachment site that contains topoisomerase II cleavage sites, J Virol 1990;64:419-23.

Capranico G, Zunino F, Kohn KW, Pommier Y. Sequence selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA binding affinity and cytotoxicity, Biochemistry 1990;29:562-9.

Nicholas AW, Wani MC, Manikumar G, Wall ME, Kohn KW, Pommier Y. Plant antitumor agents. 29. Synthesis and biological activity of ring D and ring E modified analogues of camptothecin, J Med Chem 1990;33:972-8.

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. In: Potmesil M, Kohn KW eds. DNA topoisomerases in cancer. New York: Oxford Press, 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. In: Potmesil M and Kohn KW eds. DNA topoisomerases in cancer. New York: Oxford Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CM 06170-06 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 Investigetor.) (Name, title, laboratory, and institute affiliation)*

PI:	Christopher Hatch	Senior Staff Fellow	LMPH NCI
Others:	William M. Bonner	Senior Investigator	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	0.9	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 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 and completely sequenced. The gene contains four introns and has two Alu sequences in the 5′ upstream region. There is single functional copy of the gene and one or more pseudogene copies. The expression of the gene does not vary through the cell cycle but is down-regulated as cells differentiate and enter a quiescent state. Hybridization probes specific for the functional copy of the gene are being used to determine if in different states of cell proliferation there is differential methylation of cytosine residues in the highly GC-rich promoter region and first intron. These probes are also being used to locate DNase I hypersensitive sites and thusly determine the regions of the H2A.Z gene that may be bound by transcription factors.

CAT constructs have been assembled from various fragments of the H2A.Z gene promoter region. The core promoter is located in the first 234 base pairs of sequence upstream from the start of transcription. A short stretch of sequence just upstream from the core promoter up regulates the promoter in embryonic (proliferating) cells and down-regulates the promoter in differentiated or quiescent cells. Cotransfection experiments have shown that the H2A.Z promoter is down-regulated by the HIV-TAT gene product. Experiments are in progress to determine the effect of HIV infection on histone biosynthesis.

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. There is however evidence that suggests that the basal histone variant H2A.Z comprises a larger proportion of the H2A histone in more transcriptionally active chromatin. 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. The H2A.Z gene provides an excellent model system for studying the mechanisms by which housekeeping genes modulate their level of expression according to the state of cellular proliferation and differentiation.

Methods:

1. Recombinant DNA techniques.

2. Assays of expression of transfected recombinant DNAs in mammalian cells.

3. Antisense oligonucleotides assay the effect of selective inhibition of specific histone variant expression.

4. Use of two dimensional gel electrophoresis for identification of histone variant proteins.

Major Findings and Accomplishments:

1. Isolation of Human H2A.Z Gene

The human H2A.Z gene has been isolated and completely sequenced. The coding sequence of the gene is interrupted by four introns. There is a single functional copy of the gene and one or more pseudogene copies.

2. Regulation of H2A.Z Gene Expression

The mRNA level of H2A.Z decreases 20 fold when proliferating cells become quiescent. This results from changes in both the rate of transcription of the H2A.Z gene and the stability of the transcribed mRNA. This provides us the opportunity to study the mechanisms by which the expression of the H2A.Z gene varies during different states of cell growth and proliferation.

The H2A.Z gene promoter has been delimited by cloning various lengths of upstream gene sequence in front of the bacterial chloramphenicol transferase (CAT) gene and assaying CAT activity after transfection and expression of the constructs in human cells. The core promoter has been localized to the first 234 bp of sequence upstream from the transcription start site. The 160 base pairs of sequence upstream from the core promoter appears to contain elements which up-regulate the activity of the promoter in embryonic (proliferating) cells and down-regulate the promoter in differentiated or quiescent cells. This "enhancer-silencer" region of the promoter has been cloned upstream and downstream from a heterologous promoter-reporter gene construct to test whether or not its promoter modulating ability is specific to the H2A.Z promoter. The silencer activity has been found to exert its effect on the SV40 early promoter in IMR-90 human cells that are entering the quiescent state. The enhancer activity is being assayed at present. Deletions are being made in this region of the promoter to define the cis-acting promoter element responsible for this activity.

Cotransfection experiments have shown that the H2A.Z gene promoter is down-regulated by the HIV (human immunodeficiency virus) regulatory protein, Tat. In a collaborative effort with Dr. Sandra Colombini Hatch of the Laboratory of Tumor Cell Biology we are monitoring histone biosynthesis in a number of different human lymphocytic cell lines at various times after infection with HIV. We are attempting to optimize the percent of cells either infected or transfected in order better assess the effect of viral gene expression, particularly Tat, on the expression of cellular genes such as the histones.

Proposed Course:

1. The chromatin structure in and around the H2A.Z gene is being analyzed by several different approaches. Probes specific for the functional copy of the H2A.Z gene have been isolated and are being used to determine if in different states of cell proliferation there is differential methylation of cytosine residues in the highly GC-rich promoter region and first intron. These probes are also being utilized to locate DNase I hypersensitive sites and thusly determine the regions of the H2A.Z gene that may be bound by transcription factors.

2. A collaboration is being arranged to determine the chromosomal (human) localization of the H2A.Z gene. The genes for the DNA-replication-linked histone variants are clustered on human chromosomes 1, 6, and 12. We will determine whether or not the genes for the basally-expressed histone variants are linked to each other or to the genes for the DNA-replication-linked variant forms.

3. Further characterize the switch mechanism for differential expression of the H2A.Z gene in embryonic or proliferating as opposed to differentiated or quiescent cells. Deletions and point mutations will be made in the H2A.Z gene promoter region to more precisely determine the regulatory sequences. A complementary approach will involve the use of DNase I footprinting and gel retardation assays to identify the cis-acting sequence elements and the regulatory proteins that bind to them to effect this switch.

4. Experiments are in progress to assess the effect of over- or under-expression of selected histone H2A variants in human cells. Two approaches are being utilized. First, cells will be incubated in growth medium containing antisense oligonucleotides complementary to specific stretches of sequence encoded by the H2A.Z gene and present in the mature H2A.Z mRNA. Antisense oligonucleotide design will be optimized for efficacy in reducing the steady state level of H2A.Z mRNA. The effect of reduced levels of H2A.Z gene expression on cell growth and the expression of other genes will be monitored. In particular, we will determine whether or not mechanisms are in place to couple and thusly maintain the stoichiometry of synthesis of the major and basal histone H2A variants in the transition between the proliferating and quiescent states. Second, in a parallel and complementary approach, I have cloned selected DNA fragments from the human H2A.1 and H2A.Z genes into mammalian expression vectors containing strong viral (Cytomegalovirus and Rous sarcoma virus) promoters. The initial goals are to obtain the under-expression of H2A.Z with normal levels of H2A.1, and/or the overexpression of H2A.Z with significantly reduced expression of H2A.1/2. The former situation might be obtained by the transfection, integration, and stable expression of an H2A.Z antisense gene fragment in a human cell line. The latter situation, overexpression of H2A.Z in a situation of reduced major variant H2A expression, might similarly be brought about by the cotransfection, integration, and stable expression of the H2A.Z gene and an H2A.1 antisense gene fragment. Refinements in the H2A gene fragments selected for sense or antisense expression will be made according to the initial results obtained. Additionally, systems which permit the amplification of the integrated gene fragments could be used.

Publications:

Hatch CL, Bonner WM. The human histone H2A.Z gene: sequence and regulation, J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06172-06 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters 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
	Ann Orr	Microbiologist	LMPH NCI
	Michael Walton	Visiting Fellow	LMPH NCI

COOPERATING UNITS *(if any)*

Drug Synthesis and Chemistry Branch, DTP, DCT, NCI (Drs. Ven Narayanan and Rudiger Haugwitz)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.0	0.8

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Since nitrogen mustard derivatives continue to be at the forefront of cancer chemotherapy, we are continuing to investigate the molecular mechanisms of actions of these drugs, with the view that even a modest increase in selectivity of drugs designed on the basis of new mechanistic insights could have significant clinical impact. The current focus is on nitrogen mustard derivatives containing ring systems that bind to DNA by intercalation, since this is the most direct way in which these drugs could be targetted to specific DNA regions. Intercalating mustards are also of interest because they need only a monoalkylating group for cytotoxic and antitumor activities, indicating that they are effective without forming covalent crosslinks. A homologous series of intercalating mustards is being studied as a function of the length of the hydrocarbon chain connecting the intercalating (acridine) group with a mono or a bifunctional nitrogen mustard moiety. Substantial changes in base sequence selectivity have been found depending on the connecting chain length. Current studies attempt to relate both sequence selectivity and the production of different types of DNA lesions to cell killing potency. Although we have so far studied sequence selectivity only on purified DNA, we are developing methods to study this in cell nuclei. In addition, we are studying the formation of interstrand crosslinks in synthetic oligonucleotide duplexes, in order to determine the DNA sequence dependence and the effect of drug structure. We are also studying the production and repair of DNA lesions, such as interstrand and DNA-protein crosslinds and DNA strand breaks measured by DNA alkaline elution assays, in drug-treated cells. We find that the intercalating mustards are unusual, compared to the common nitrogen mustards, in that DNA strand break production is prominent and interstrand crosslinks are not detected. Intercalating mustards therefore may have a unique mechanism of action.

PROJECT DESCRIPTION

Objectives:

1. Determine the structural bases of observed nucleotide sequence selectivities of the reactions of alkylating agents with DNA.

2. Determine the DNA sequence selectivities for the reactions of alkylating agents in intact cells or isolated nuclei.

3. Determine the DNA sequence dependence for interstrand crosslink production.

4. Design new alkylating agents having optimized DNA sequence selectivities, and investigate the possibility that this could lead to enhanced antitumor specificities.

5. Determine the kinetics of formation and repair of DNA lesions in relation to cell survival. The DNA lesions of interest for which methodology is available include DNA interstrand and DNA-protein crosslinks and DNA strand breaks.

Methods:

1. Quantitative high resolution gel electrophoresis to determine selectivity of alkylation as a function of DNA sequence. Radioactivity in electrophoretic gels is determined using a Betascope (Betagen Corp.) radioactivity analyzer, and data are analyzed using special purpose computer programs that we have developed.

2. Polyacrilamide gel electrophoresis of oligonucleotides to resolve alkylated and crosslinked species.

3. DNA alkaline elution assays for the determination of DNA strand breaks, interstrand crosslinks and DNA-protein crosslinks in cells.

4. Clonogenic assay of survival of drug-treated cells.

5. Standard methods of cell and molecular biology.

Major Findings:

1. Intracellular pharmacokinetics of DNA lesion formation and repair for nitrogen mustards.

In a previous study of the DNA sequence selectivity of guanine-N7 alkylation by various nitrogen mustards, we had found that uracil mustard differs from several other common nitrogen mustards, in that it often reacts strongly in the sequences of the type 5′-GC-3′, where other nitrogen mustards generally react very weakly. Since 5′-GC-3′ was thought to be the sequence where interstrand crosslinks are most likely to occur, we asked whether uracil mustard, compared to other nitrogen mustards, may be more efficient in producing

these DNA lesions. Although the answer to this question was negative, it led us to take seriously the possibility, now supported by work described in a later section of this report, that 5′-GC-3′ sequences are, in fact, not the major sites of interstrand crosslinking.

In carrying out the above study, we obtained data that we view as intracellular pharmacokinetics, in the sense that molecular lesions at intracellular target sites might be considered as compartments in a comprehensive model. We have obtained quantitative estimates for several common nitrogen mustards which relate extracellular drug concentration to the kinetic constants for the formation and removal of DNA interstrand crosslinks and DNA-protein crosslinks. Cell killing by several nitrogen mustards was found to depend in a simple manner on the area under the interstrand crosslink frequency versus time curve.

An exception to the above rule, however, was quinacrine mustard. The nitrogen mustard group in this compound is connected to a quinacrine ring system which binds to DNA by intercalation between base pairs. We had previously determined the unique base sequence preferences for alkylation by the mustard group, which are attributable to the intercalation of the quinacrine group. An unexpected new finding was that, contrary to most mustards, which produce extensive interstrand crosslinking without detectable DNA strand breakage, quinacrine mustard produces extensive strand breakage without detectable interstrand crosslinking. This observation, together with our previous findings on the unusual DNA sequence preferences, led us to initiate more detailed studies of intercalating mustards.

2. DNA intercalating mustards.

Through the cooperation of the Drug Synthesis and Chemistry Brance, a homologous series of intercalating nitrogen mustards are being synthesized under contract with Dr. Frank S. Guziec at the University of New Mexico). The compounds contain an acridine ring system as the intercalating moiety which is connected by a straight hydrocarbon chain to a mono or a bifunctional nitrogen mustard group. The plan is to compare the mono and bifunctional compounds as a function of the length of the connecting chain. The monofunctional compounds are of special interest, because they may represent a mechanistically novel type of agent which is chemically unable to produce crosslinks. Indeed, monofunctional compounds of this type have been reported to be active in some murine tumor systems.

To date, 12 of the 18 planned compounds have been synthesized and received in our laboratory, and we have at least preliminary experimental data on most of them. We are studying the effects of connecting chain length on (1) DNA sequence selectivity in purified DNA, (2) the formation of interstrand and DNA-protein crosslinks in cells and (3) the cytotoxicity ratios for the corresponding mono and bifunctional derivatives.

We have found that the DNA selectivity depends in a coherent manner on connecting chain length. Short chain lengths of 2 or 3 carbons produced the greatest selectivity, and tended to confine reaction primarily to 5′-GG-3′ sequences. This is of considerable interest, because such sequences occur in regulatory regions of certain genes, including oncogenes. Long chain lengths

in the range of 5 or 6 carbons generally reduced the selectivity, and allowed substantial reaction at many sites that are nearly free of attack by compounds having shorter chain lengths. It will now be possible to test whether DNA sequence selectivity influences cell killing.

In cells, a short chain length monofunctional derivative produced DNA strand breaks as the only DNA lesion detectable by DNA alkaline elution. This clarifies the findings already mentioned for quinacrine mustard. The cytotoxicities of corresponding mono and bifunctional compounds are being compared. Preliminary data on the killing of human colon tumor HT-29 cells indicated an optimum potency at chain lengths of 3 or 4. The monofunctional compounds were only moderately less potent than the corresponding bifunctionals, suggesting that the cytotoxicity of these compounds is largely attributable to DNA lesions other than crosslinks.

3. DNA interstrand crosslinking in synthetic oligonucleotide duplexes.

As already noted, the failure of uracil mustard to exhibit the expected enhancement of interstrand crosslink production put into question the previously accepted assumption that these lesions would occur primarily at 5′-GC-3′ sequences. We have therefore set up methodology to study DNA interstrand crosslinking in oligonucleotide duplexes of defined sequence. Our experiments so far confirm the previously reported production of interstrand crosslinks by HN2 surprisingly at 5′-GNC-3′, rather than at 5′-GC-3′, sequences. Uracil mustard however did produce interstrand crosslinks in 5′-GC-3′ sequences. In line with the earlier DNA sequence preference data, addition of a methyl group to the 6 position of uracil mustard, which abolishes the unusual DNA sequence preference, causes interstrand crosslinking of oligonucleotide duplexes to revert primarily to 5′-GNC-3′ sequences. Thus it appears that uracil mustard does in fact have an unusually strong ability to form interstrand crosslinks at 5′-GC-3′ sequences, as we had predicted.

Proposed Course:

1. Extend the DNA sequence selectivity studies to DNA in cells or isolated nuclei, in order to determine to what extent the rules derived for purified DNA may or may not apply. To do this, a polymerase primer extension method is being developed.

2. Complete the structure-activity studies of intercalating mustards in purified and cellular systems.

3. Continue the investigation of the DNA sequence determinants for interstrand crosslink production in defined oligonucleotide duplexes.

4. Investigate the possibility that certain G-run sequences in the control regions of certain genes may constitute hot-spots for alkylation reactions.

5. Study the structure of intercalating mustard DNA complexes by computer molecular modelling, and propose new candidate structures for synthesis in order to test hypotheses on the determinants of sequence selectivity.

Publications:

O'Connor PM, Kohn KW. Intracellular pharmacokinetics of DNA lesion formation and repair by nitrogen mustard, phenylalanine mustard, uracil mustard, 6-methyluracil mustard and quinacrine mustard. Cancer Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CM 06186-04 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

DNA Damage and Repair at the Level of the Gene

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, leboratory, end institute affiliation)*

PI:	Vilhelm A. Bohr	Medical Officer	LMPH NCI
Others:	Alfred May	Microbiologist	LMPH NCI
	Karsten Wassermann	Visiting Fellow	LMPH NCI
	Michele Evans	Medical Staff Fellow	LMPH NCI
	Jennifer C. Jones	Student, Gift Fund Fellow	LMPH NCI
	Tinna Stevnsner	Guest Researcher	

COOPERATING UNITS *(if eny)*

Dr. Rodney Nairn (MD Anderson); Dr. Jay Robbins (NIH); Dr. Michael Gottesmann (NIH); Dr. Snorri Thorgeirsson (NIH); Dr. Michael Potter (NIH)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.7	3.7	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Our objective is to study the DNA damage and repair in genes and other regions within the genome. We also use more traditional techniques to study DNA repair in the average, overall genome. Findings (reviewed in Bohr VA et al., Laboratory Investigation 61, 143, 1989), 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 end-points and risk assessments. Whereas our earlier studies were limited to UV as a damaging agent, we have now developed techniques to study DNA damage and repair after certain carcinogens and cancer chemotherapeutics.

We are studying the DNA repair of genes in some human, cancer prone DNA repair deficient syndromes and in various human and rodent mutant cell lines, some of which are transfected with repair genes. We are also investigating the role of DNA repair in multidrug resistance.

Professional Personnel (Continued):

Weiping Zhen	Visiting Fellow	LMPH NCI
Jeffrey Beechman	Graduate Student	LMPH NCI
Charles Link	Medical Staff Fellow	LMPH NCI

Objectives:

1) To study DNA damage and repair in specific genes using established methodology. To develop new methodology to examine DNA damage and repair in structural genes and oncogenes following treatment with various carcinogens and cancer chemotherapeutics.

2) Examine DNA repair in important genes in DNA damage sensitive human cell lines and in various rodent cell lines.

3) Investigate the role of DNA repair in the overall genome and in specific genes in multidrug resistance.

4) Further explore the molecular rules that govern preferential repair of genes in normal and repair deficient mammalian cells.

Methodology:

Cell culture techniques. DNA damage and repair. Drug pharmacology. DNA molecular techniques, molecular hybridizations. RNA techniques. Hybridization and quantitation. General molecular biology. Centrifugation techniques. Densitometry. Plasmid analysis. 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. In normal human cells, we have found that genes are repaired faster than the bulk of the genome. It appears that determinations of DNA repair in specific genomic sequences may be more important than overall genome DNA repair measurements when we wish to correlate repair with 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 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. When metallothionein genes which are normally inactive become activated, considerable corresponding increases in DNA repair efficiencies can be detected. These findings suggest that there is a positive correlation between DNA repair efficiency and transcriptional activity in a gene.

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 precise 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 naevus syndrome, Bloom syndrome, Cockaynes syndrome and Gardiner'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 possibly in Gardiner's syndrome. The lack of preferential DNA repair in the latter two diseases is of considerable interest, since it is the first example of human cells that are 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 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 UV induced adducts other the pyrimidine dimer. We are currently examining the repair of other adducts in the DHFR gene in these human repair deficient cells. In XPA we found some repair of the DHFR gene; this is of interest since previous reports on UV repair in this cell line (only done at the level of the overall genome) shows no repair at all. Our findings thus suggest that there is repair activity in the cells, and that the limited activity may be directed towards vital regions.

2. Effect on gene specific 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 repair in CHO cells after UV damage. The DNA polymerase alpha 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 inhibited the overall genome repair by about 30%, but it did not affect the repair in the DHFR gene. The topoisomerase I inhibitor campthothecin 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. Although these inhibitor experiments did not reveal a preferential involvement of any enzyme in the repair of active gene, we find that when a topoisomerase I inhibitor (camptothecin) and a topoisomerase II inhibitor (merbarone) both are added, the gene specific repair is strongly inhibited. Those studies suggest that topoisomerases are involved in gene specific repair, but that one type can take over for the other.

3. Formation and repair of minor photoproducts in the DHFR gene.

UV irradiation introduces photoproducts in the DNA; the major photoproduct (60-80% of total) 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. It appeared that the frequency of 6-4 PP is about 40% of that of pyrimidine dimers in the CHO DHFR gene. This frequency is higher than previously thought, and that may be important for our thinking about mutations formed by this adduct. We found that the 6-4 PP is preferentially repaired in the gene as compared to non-transcribed genomic regions. We are now using this assay to examine the formation and repair of 6-4 PP in the DHFR gene in normal human cells and in cells from various complementation groups of xeroderma pigmentosum.

4. DNA damage and repair in genes after treatment with carcinogens and chemotherapeutics.

With the use of the ABC excinuclease, we can now quantitate cisplatinum, 4NQO, IQ (a highly mutagenic, heterocyclic compound) and NAAAF adducts in individual genes. We find preferential DNA repair of cisplatinum adducts in the CHO DHFR, whereas NAAAF adducts are not preferentially repaired. In addition we now have an assay to directly detect the sites of alkylation damage in DNA. In this assay, the DNA is nicked at N7-alkylated sites by depurination and endonucleolytic cleavage, and the frequency of alkylated sites in genes are probed. We have recently found that adducts formed after treatment with nitrogen mustard are initially formed at different adduct frequencies in different genomic regions and that they are preferentially repaired in the CHO DHFR gene. Cisplatin adducts can be quantitated within genes using the ABC excinuclease, and we can detect cisplatin interstrand crosslinks using a denaturation-reannealing gel assay. For both of these lesions, we find preferential repair in the DHFR gene as compared to inactive genomic regions.

5. Multidrug resistance and DNA repair.

Many mechanisms are known to play a role in drug resistance, and lately the DNA repair processes have been in focus. Far a number of resistant mammalian cell lines it has been shown that DNA repair is more efficient than in the paternal cell line. However, the degree of increased drug resistance is higher than the increase in DNA repair. But all of these repair studies have been done only at the overall genome level. We have begun to study cisplatin adduct and crosslink removal in specific genes in resistant and sensitive human cell lines. In two human ovarian cancer cell lines, we find that there is a similar level of cisplatin adduct repair in the DHFR gene in the sensitive and resistant cells. But when we examine the levels of interstrand crosslinks in the gene, there is increased level of repair in the resistant cells compared to the sensitive.

6. DNA repair in the murine c-myc locus.

We have studied DNA repair after UV damage in the murine c-myc locus. It appears that a region in B cells upstream of the murine c-myc gene is repaired with a different efficiency in plasmacytoma-resistant DBA/2N mice than in plasmacytoma-susceptible BALB/cAn mice. The region just upstream of c-myc is inefficiently repaired in B lymphoblasts derived from BALB/cAn mice. In contrast, this same region of c-myc is efficiently repaired in B lymphoblasts derived from DBA/2N mice. DNA fragments located in the coding region of c-myc and in another gene, dihydrofolate reductase (DHFR), are repaired with equal efficiency in cells from these two strains of mice. It is possible that repair efficiency of the 5′ flank of c-myc may be involved in tumor susceptibility of the mouse strain.

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 two overall directions in our research program: 1) the further investigations of the mechanism of preferential DNA repair, and 2) gene-specific DNA repair in human disease, the relation to cancer risk and therapy.

Mechanisms 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 mechanism of gene-specific repair. They include the influence on repair efficiency in a gene of 1) the transcriptional state or level of a given gene, 2) the "type" of gene (essential, structural, regulated, stage of differentiation), 3) a number of chromatin structural elements (accessibility, ADP ribosylation, methylation) and 4) the type of topoisomerase and DNA polymerase involved. We are currently investigating some of these relationships: We are assessing the repair in some genes, some of which can be modulated with regard to transcriptional activity and some of which are at different developmental or differentiational stages. We are interested in the question of whether genes are repaired by specific enzymes different from those involved in the repair of the overall genome. To search for the under-

lying genetics of the gene-specific repair, we are examining the repair in CHO mutant cell lines and in CHO cells transfected with some of the recently cloned human DNA repair genes. All the above studies will initially be done using UV irradiation as the damaging agent. Important findings will be followed up using alkylation damage or cisplatin.

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 studying the repair in different genes in selected 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 Gardiner's syndrome.

We are studying the repair in a number of important genes including the MDR-1 gene, the topoisomerase II gene, the poly ADPribose gene and the DNA polymerase beta 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:

Thomas DC, Okumoto DS, Sancar, A, Bohr VA. Preferential DNA repair of (6-4) photoproducts from the CHO DHFR gene, J Biol Chem 1989;264:18005-10.

Bohr VA, Evans MK, Fornace AJ Jr. DNA repair and its pathogenetic implications, Lab Invest 1989;61:143-61.

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 1989;264:14455-62.

Bohr VA, Evans MK. DNA repair in individual genes. Implications for oncology, J Pediatr Oncol 1990;7:47-69.

Bohr VA. DNA repair at the level of the gene: molecular and clinical considerations, Cancer Res Clin Oncol, in press.

Parker, RJ, Poirier MC, Bostick-Bruton F, Vionnet J, Bohr VA, Reed E. The use of peripheral blood leukocytes as a surrogate marker for cisplatin drug resistance - studies of adduct levels and ERCC1. Brookhaven Symp in Biology, no. 36, DNA damage and repair in human tissues, in Press.

Link CJ Jr, Bohr VA. DNA repair and drug resistance. Studies on the repair process at the level of the gene. In: Mcguire, WL, Ozols, RF, eds. In Drug Resistance. 2nd ed. Norwell: Kluwer Acad Publ,in press.

Wassermann K, Kohn KW, Bohr VA. Heterogeneity of nitrogen mustard induced damage and repair at the level of the gene following treatment of CHO cells, J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CM 06192-02 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

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:
1.8	1.3	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 primary objective of this project is to isolate the cDNA and gene for human histone H2A.X, and study the regulation of this protein and its mRNA in proliferating and quiescent cells.

During the previous year, we isolated the cDNA for human H2A.X. This H2A related protein which had not been sequenced previously yielded some intriguing information concerning the relationship of the H2A histone species during evolution. Seven of the final carboxy-terminal amino-acids of human H2A.X 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.

The mRNA is very unusual in that it contains stem-loop and U7 snRNP sequence motifs found in mRNAs from replication-dependent histone species, as well as polyA motifs. Two mRNAs exist in proliferating cultures, one terminating at the stem-loop motif and the other at the polyA motifs with a polyA tail. Later this year, a new postdoctoral fellow will being studies of the mechanism of this regulation.

We have isolated a gene for H2A.X from human genomic DNA. Results of Southerns suggest that there is only one gene for this protein and that the H2A.X gene does not contain introns.

These results suggest that the regulation of histone biosynthesis may be more complex than previously thought, with transcripts from the same mRNA being processed for histone synthesis in both proliferating and quiescent cells. Synthetic oligos may be useful in inhibiting this processing in a therapeutically useful manner.

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. We found that H2A.X contains a carboxy-terminal motif conserved with those of lower eukaryotes, suggesting that H2A.X is a more primitive histone sequence. The H2A.X transcript was also found to be unique in that it is processed alternatively to yield both replication-dependent and independent mRNAs.

Objectives:

The current objectives of this project are (1) to isolate the H2A.X gene, (2) to compare its sequence, particularly in the promoter region to those of other H2As, (3) to prepare artificial genes to study in vivo and in vitro the factors which determine the ratios of replication-dependent and independent transcripts from the H2A.X gene.

Methods:

1. Recombinant DNA techniques.
2. Construction of artificial genes and transfection into cells.
3. Use of two dimensional gel electrophoresis for identification of histone isoproteins.

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 was 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. Isolation and partial Characterization of the H2A.X Gene

Using probes for the 3′ and 5′ UTR regions of the H2A.X cDNA, we have isolated

some clones from a human genomic library in lambda. These clones are currently being subcloned for sequencing. One clone has been partially characterized. It contains about 0.5 kb upstream from the CAP site and 1 kb downstream from the polyA site. The 5′ upstream region of the H2A.X gene is situated near one end of this genomic fragment; however, we also have isolated other genomic fragments that should contain more upstream sequence should we feel we need it. This H2A.X gene does not seem to contain any introns.

3. 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 mRNA contains both types of sequences, and two mRNAs are present in proliferating cells. There is considerable variation in the ratio of the two mRNA forms. In Jurkat cells, there is much more short form mRNA than long form, while in proliferating IMR-90 cells, there is more long form than short form. In quiescent IMR-90 cells, the short form is not visible, and the long form is also present in decreased amount. The significance of these differences is currently under study.

Significance to Biomedical Research and to the Program of the Institute

Through the use of various synthetic oligonucleotides, it may be possible to disrupt the coordination of replication-dependent histone and DNA synthesis in a therapeutically useful manner. In addition, 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 H2A.X mRNA. 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 and use it to prepare artificial genes to study the factors involved in the regulation of the mRNA processing.

3. Use synthetic oligos to disrupt the regulation of H2A.X mRNA processing.

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 1989;17:9113-26.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07102-15 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 charecters 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	LMPH, NCI
Others:	R. Bai	Visiting Associate	LMPH, NCI
	Z. Getahun	Visiting Fellow	LMPH, NCI
	G. J. Kang	Staff Fellow	LMPH, NCI
	S. Grover	Visiting Fellow	LMPH, NCI
	C. M. Lin	Biologist	LMPH, NCI

COOPERATING UNITS *(if any)* 1) 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. Nguyen, FDA; 7) Dr. J. B. Jiang, Dupont Corp.; 8) Dr. S. Friedman, LBC, DTP, DCT, NCI

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	3.2	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.)*

The goal of this project is the development of new antineoplastic agents directed against tubulin, a protein critical for cell division and for the maintenance of cellular morphology. Detailed mechanistic and structure-activity studies were performed with seven classes of compounds. Combretastatins A-2 and A-4 were found to bind to tubulin rapidly and reversibly, but with high affinity, at the colchicine site, and studies to determine the optimal bridge length between phenyl rings were initiated. The interaction with tubulin of the pentapeptide dolastatin 10, a noncompetitive inhibitor of vinca alkaloid and nucleotide binding, was defined with great precision based on properties of stereoisomers and segments of the active molecule. Mechanistic studies were initiated with dolastatins 11, 14, and 15, which do not appear to interact with tubulin in vitro but which disrupt the function of cellular microtubules. Structure-activity and mechanistic studies were continued or initiated with analogs of colchicine, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone, derivatives of 2-strylquinazolin-4-one (SQZ), and derivatives of 5,6-diphenylpyridazin-3-one (DPP). The SQZ derivatives were defined as compounds binding rapidly and reversibly, but with low affinity, at the colchicine site. The DPP derivatives appear to bind at a unique binding site on tubulin. The evaluation of the mechanism of action of the antimitotic agent 2,4-dichlorobenzyl thiocyanate was completed with the demonstration that it formed a mixed sulfhydryl with ß-tubulin by alkylating cysteine residue 239.

Cooperating Units (Continued):

9) Dr. G. I. Kingston, Virginia Polytechnic Institute and State University

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 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. Studies are in progress to define the optimum length of the hydrocarbon bridge between the two phenyl rings of the combretastatins.

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 radiolabeled 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 eighteen stereoisomers of the peptide, several of which are active, and one active tripeptide segment. The tripeptide segment inhibited tubulin polymerization and the associated GTPase reaction, but, unlike dolastatin 10, it had little effect on either nucleotide exchange or vinca binding to tubulin. The combination of the isomer studies and the properties of the tripeptide segment permitted us to propose a unique model for dolastatin 10 binding to tubulin. The peptide probably binds to ß-tubulin at a site distinct from the vinca and exchangeable nucleotide sites but in close proximity to these sites. The key residue for interaction of dolastatin 10 with tubulin is its third amino acid, termed dolaisoleuine, which is probably derived from isoleucine.

Besides dolastatin 10, Dolabella auricularia produces three highly cytotoxic depsipeptides named dolastatins 11, 14, and 15. Unlike dolastatin 10, these three depsipeptides do not have significant effects on in vitro biochemical properties of tubulin; but they do appear to interfere with normal microtubule function in cells. Dolastatins 14 and 15 cause a rise in the mitotic index of L1210 murine leukemia cells, while dolastatin 11 causes the appearance of high numbers of multinucleated L1210 cells. Normally bipolar glial cells treated with dolastatin 11 become multipolar with multiple processes containing what appear to be thick bundles of microtubules. In collaboration with Dr. S. Friedman of the LBC, DTP, DCT, NCI we are evaluating the effects of the D. auricularia peptides and depsipeptides on cell morphology and the distribution of cytoskeletal components, in particular the microtubules. The initial findings just summarized are unique in that thus far no potent antimitotic agent (one which inhibits cell growth at submicromolar concentrations) has been described which does not interfere with tubulin polymerization and in that a single species may produce agents which both inhibit and enhance microtubule assembly.

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 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 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 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 demonstrating interaction of these compounds by equilibrium chromatography (by the Hummel-Dreyer technique). Quantitative evaluation of the binding parameters of the two radiolabeled compounds will be performed, and they will then be used to demonstrate whether or not this class of agents binds at a unique site on tubulin.

6) Dr. J. B. Jiang of the Dupont Corporation has synthesized a series of derivatives of 2-styrylquinazolin-4-one (SQZ) with antineoplastic activity. The most active members of the series strongly inhibit tubulin polymerization, and we performed a structure-activity analysis of the approximately forty available analogs. The entire SQZ backbone is required for optimal antitubulin activity, and substituents were only well-tolerated at position 6. Activity of SQZ derivatives was enhanced if the position 6 substituent was either a halide atom or a small hydrocarbon group (methyl, methoxy, acetyl). There was good correlation between antitubulin activity and cytotoxicity of this class of compounds, and active agents caused the accumulation of cells arrested in mitosis. Studies are now underway to define the mechanism of binding to tubulin. Preliminary data indicate that these agents bind at the colchicine site (competitively with colchicine), but that their binding and dissociation reactions with tubulin occur extremely rapidly.

7) The compound 2,4-dichlorobenzyl thiocyanate (DCBT) had previously been shown to form multiple mixed disulfides with cysteine residues of tubulin, thereby inhibiting tubulin polymerization and causing cells to accumulate in mitosis. We showed, in collaboration with Dr. N. Y. Nguyen of the FDA that the most sensitive cysteine residue was that which occurs at position 239 in ß-tubulin.

8) Dr. D. G. I. Kingston of Virginia Polytechnic Institute is presently synthesizing photoactive analogs of taxol. We plan to characterize the biological activities of these analogs with the specific goal of using them to define the binding site of taxol on tubulin. Covalently modified tubulin peptides will be isolated in our laboratory and sequenced by Dr. Kingston.

Publications:

Bai R, Lin CM, Nguyen NY, Liu T-Y, Hamel E. Identification of the cysteine residue of ß-tubulin alkylated by the antimitotic agent 2,4-dichlorobenzyl thiocyanate, facilitated by separation of the protein subunits of tubulin by hydrophobic column chromatography, Biochemistry 1989;28:5606-12.

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Hamel E. Interactions of tubulin with small ligands. In: Avila J ed. Microtubule proteins. Boca Raton: CRC press, 1990;89-191.

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Bender RA, Hamel E, Hande KR. The plant alkaloids. In: Chabner, BA, Collins JM, eds. Cancer chemotherapy: principles and practice. Philadelphia: JB Lippincott, 1990;253-75.

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Jiang JB, Hesson DP, Dusak BA, Dexter DL, Kang GJ, Hamel E. The synthesis and biological evaluation of 2-styryl quinazolin-4-(3^H-ones, a new class of antimitotic anticancer agents which inhibit tubulin polymerization. J Med Chem, 1990;33:1721-28.

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Bai R, Pettit GR, Hamel E. Structure-activity studies with chiral isomers and with segments of the antimitotic marine peptide dolastatin 10. Biochem Pharmacol, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07179-05 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

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	LMPH, NCI
Others:	R. Bai	Visiting Associate	LMPH, NCI
	Z. Getahun	Visiting Fellow	LMPH, NCI
	G. J. Kang	Staff Fellow	LMPH, NCI
	S. Grover	Visiting Fellow	LMPH, NCI
	C. M. Lin	Biologist	LMPH, NCI

COOPERATING UNITS *(if any)*
Dr. P. Skehan, PDRG, DTP, DCT, NCI

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	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 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 alpha-tubulin and ß-tubulin on a preparative scale. The purification of a microtubule-associated protein which causes the formation of microtubule bundles continued to progress and a project 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. In particular, major differences in effects of Mg^{2+} and Be^{2+} on tubulin polymerization, tubulin precipitation, polymer stability, and nucleotide binding and hydrolysis were evaluated in detail. A project to define the disulfide bridges in tubulin and in two major classes of microtubule-associated proteins was nearly completed, with the observation that all cysteine sulfhydryl groups in these proteins were free.

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 during polymerization and can only be removed from tubulin 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 findings 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.

We have therefore examined in detail the effects of Be^{2+} on polymerization with MAPs, including polymer morphology (in collaboration with Dr. P. Skehan of the PDRG, DTP, NCI), polymer stability, nucleotide binding, and nucleotide hydrolysis. This alternate cation had remarkable effects on these properties, in comparison to effects of the physiological cation Mg^{2+}. The Be^{2+} polymer was much more stable than the Mg^{2+} polymer to cold depolymerization, and it consisted of ribbons of parallel protofilaments as opposed to normal microtubules. Be^{2+}, unlike Mg^{2+}, did not enhance the binding of GTP to tubulin-GDP, but it did not interfere with the exchange of GDP bound to tubulin for GDP in the medium. In addition, Be^{2+} profoundly inhibited GTP hydrolysis, with the polymer formed in its presence containing large amounts of unhydrolyzed exchangeable site GTP. Further, relatively low concentrations of $Be2^{+}$ quantitatively precipitated tubulin at low temperatures. If both Mg^{2+} and Be^{2+} were in the reaction mixture, the nucleotide reactions more closely resembled those occurring in the presence of Mg^{2+} alone (polymerization closely coupled to GTP hydrolysis, with polymer containing little unhydrolyzed GTP, while the precipitation of tubulin and stabilization of polymer were largely unchanged. Other cations were also examined for effects on these reaction, and several others also presented unique patterns. With zinc, for example, tubulin precipitation and cold stabilization of polymer were observed, but the polymer contained only hydrolyzed GTP.

We explain these findings with a model which envisages two distinct reactions which occur in the presence of Be^{2+}. We postulate that low affinity cation

sites, which interact poorly with Mg^{2+}, on tubulin are responsible for the stabilization of polymer and the precipitation of tubulin which occur with $Be2^{+}$.

In addition, we believe that Be^{2+} probably does replace Mg^{2}+ in enhancing the binding of GTP to tubulin. We have other data which suggest that the Mg^{2+} is released from tubulin once the nucleotide is bound, leading to the idea that the cation is required for optimal GTP binding but not subsequent polymerization and GTP hydrolysis. We therefore postulate that following binding of Be^{2+}-GTP to tubulin, the cation is not released and interferes with subsequent GTP hydrolysis.

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 requires 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 unabiguously the essential triphosphate.

We plan to exploit the known ability of guanosine 5′-[alpha-ß-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 alpha-ß 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 have utilized the ability to separate the alpha-and ß-tubulin subunits in a project to locate the protein's reported cystine disulfide bridges. Contrary to the literature, which described two cystine bridges in each alpha-ß tubulin dimer, we find that there are no disulfide bonds in tubulin, despite a cysteine content of twenty residues in the dimer (eight in ß, twelve in alpha). In addition, we have fond no evidence for disulfide bonds in MAPs fractions enriched for tau protein or MAP-2.

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

Bai R, Lin CM, Nguyen NY, Liu T-Y, Hamel E. Identification of the ysteine residue of ß-tubulin alkylated by the antimitotic agent 2,4-dichlorobenzyl thiocyanate, facilitated by separation of the protein subunits of tubulin by hydrophobic column chromatography, Biochemistry 1989;28:5606-12.

Hamel E. Interactions of tubulin with small ligands. In: Avila J, ed. Microtubule proteins. Florida: CRC Press, 1990;89-191.

Hamel E, Lin CM. A reexamination of the role of nonhydrolyzable guanosine 5′-triphosphate analogues in tubulin polymerization: reaction conditions are a critical factor for effective interactions at the exchangeable nucleotide site, Biochemistry 1990;29:2720-29.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 07184-01 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Biology of Cellular Injury

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

PI:	A. J. Fornace, Jr.	Senior Investigator	LMPH NCI
Others:	M. Papathanasiou (departure 7/1/90)	Visiting Fellow	LMPH NCI
	I. Alamo	Microbiologist	LMPH NCI
	M. C. Hollander	Microbiologist	LMPH NCI

COOPERATING UNITS *(if any)* NIA, Baltimore, MD (N. Holbrook); Univ. of Cinncinati (D. Nebert); Oxford Univ., U.K. (I. Hickson); University of Utah, Salt Lake City, UT (L. Barrows); Wash. Univ., St. Louis, MO (A. Laszlo); Lawrence Livermore Natl. Lab., Livermore, CA (L. Thompson); MD Anderson, Univ. of Texas, Smithville, TX (R. Nairn); N.C.I. (J. Robbins); Imperial Cancer Research Fund Lab., London, U.K. (G. Stark); Georgetown Univ. (M. Smulson).

LAB/BRANCH
Laboratory of Molecular Pharmacology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.15	1.0	1.15

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.)*

This project is a continuation of projects Z01 CM 06380-03 RO and Z01 CM 06365-06 RO, which were initiated in the Radiation Oncology Br., C.O.P., D.C.T., N.C.I. Both of the former projects dealt with the study of stress responses in mammalian cells. The first project involved the isolation and characterization of DNA-damage-inducible (DDI) genes. The second project involved the study of heat-shock genes, which are currently being used as a model for more general stress response genes that are not specific for DNA damage. In bacteria and yeast, many of the genes important in the cellular response to DNA damage are induced by such damage. Until recently, evidence for such specific responses to genotoxic stress in mammalian cells was not definitive. Our group has been in the forefront in demonstrating that certain genes in mammalian cells can be specifically induced by specific types of DNA damage and that SOS-like responses exist in mammalian cells. Using specialized cloning procedures which we developed, cDNA clones for more than 20 novel DDI genes have been isolated and sequenced. Based on sequence analysis and other studies, we have evidence that at least one encodes a nucleic acid binding protein and that a second may play a role in DNA replication. Five of our DDI genes were found to be coordinately induced by either DNA damage or inhibition of cell growth. There is good evidence in both bacteria and eukaryotes that inhibition of cell growth after DNA damage can have a protective effect; e.g., one of the SOS genes is a growth arrest gene. In collaboration with D. Nebert, we have found that these genes were coordinately overexpressed in a mouse mutant which may provide insight into their regulation. Two of these genes have been sequenced and newly-described regulatory regions have been identified and partially characterized; antibodies to one of the proteins have been developed in collaboration with N. Holbrook. In collaboration with G. Stark, I. Hickson, and L. Barrows, expression of our DDI transcripts in DNA repair mutants has been investigated. Several examples of both increased and decreased expression in the mutant cells has been observed.

GPO 914-918

Project Description

Objective: To isolate DNA-damage-inducible genes in mammalian cells and to study both their function and regulation.

Methods Employed

Standard molecular biology techniques and specialized hybridization subtraction cDNA cloning approach which was developed in this laboratory.

Major Findings

Isolation of more than 20 different cDNA clones that code for DDI transcripts including many that were specifically induced by DNA damaging agents. Many of these genes have been well-conserved (which suggests important functions) since they were expressed and induced in human cells. One cDNA clone, DDIA18 was found to code for a nucleic acid single-stranded binding protein which is DNA-damage inducible. The human equivalent of DDIA18 has been isolated and sequenced; this protein is very highly conserved in humans and rodents. Several examples of abnormal expression were found in DNA repair mutants.

Genes for several of our growth arrest DDI clones have been isolated; and two have been sequenced. These genes have been named the *gadd* genes for growth-arrest and DNA-damage inducible. Based on studies done in collaboration with D. Nebert, we have good evidence that these genes are coordinately regulated. In addition, the promoters of the *gadd45* and *gadd153* genes contain areas of significant sequence homology. These areas of homology do not correspond to known transcription factor recognition sequences, and thus it is likely that one or more new transcription factors may be involved in the regulation of these genes. Using CAT-reporter gene constructs, we have found that regions in the promoter of the *gadd45* gene are DDI, and, in collaboration with N. Holbrook, we have also identified DNA damage responsive elements in the *gadd153* gene. We have isolated and sequenced full-length cDNA clones for both human and hamster *gadd45* and *gadd153*. Interestingly, both sequences have been highly conserved in the 2 species. Antibodies for the gadd153 protein have been developed and efforts are underway to isolate antibodies for gadd45 protein. Preliminary experiments indicate that the gadd153 protein is localized to the nucleus.

The *gadd45* gene is particularly interesting since we have found that it is strongly induced by x rays in human cells. Since this gene was not induced by inducers of protein kinase C, it represents the first x-ray-inducible human gene that is induced by a protein kinase C-independent mechanism. In collaboration with J. Robbins, we have found that the induction of this gene is significantly reduced in cells from patients with ataxia telangiectasia, a disease characterized by radiosensitivity and increased cancer risk.

In the case of the heat-shock genes, we have isolated the largest collection of cDNA clones for such genes in mammalian cells; this includes clones encoded by more than 15 different heat-shock genes. We have found that, in addition to being induced by hyperthermia, some heat-shock genes are induced by certain DNA-damaging agents. For example, ubiquitin was induced by alkylating agents and hsp27 by UV-type agents. In collaboration with L. Barrows, hsp70 mRNA was found to be induced in hamster cells by BCNU; the magnitude of the induction varied in cells with differing sensitivity to alkylating agents. In the case of hsp27, we explored the relation of the expression of this gene to cellular sensitivity to UV radiation and similar DNA-damaging agents. Using cells developed by J. Landry (Landry *et al*, J. Cell. Biol. 109, 7, 1989) that contain a hsp27

expression vector, we have found that cells with increased expression of this gene show increased resistance to the UV lethality. Thus, increased expression of a gene, which is DDI, is associated with increased resistance to such agents. Another heat-shock gene study involves the expression of these genes in heat-sensitive thermotolerant-defective cells. We have found that the induction of many heat-shock genes is altered in one such mutant isolated by Harvey and Bedford (Radiation Res. 113, 526, 1988). In this mutant heat-shock gene transcripts increase in abundance after heat-shock, but remain at elevated levels much longer than normal. In collaboration with A. Laszlo, we have found that protein synthesis is depressed in these cells after heat-shock and thus the over-expression of the heat-shock genes may reflect a lack of normal negative feedback control.

We have also initiated studies on the effect of aging on stress response genes in collaboration with N. Holbrook. Initial studies involved the expression of hsp70 mRNA in primary cultures freshly isolated from old and young rats. Induction of hsp70 mRNA was significantly reduced in cells from the older animals. These studies will be expanded to other stress response genes with emphasis on DDI genes.

Significance to Biomedical Research and the Program of the Institute

DNA damage and its repair play a central role in the cellular response to many antineoplastic agents and also in carcinogenesis. Since our clones code for genes induced by DNA damage, it is likely that their protein products play a role(s) in the response of cells to this type of injury.

Proposed Course

We plan to concentrate on the study the regulation of the *gadd* genes and the functions of their protein products. Attempts will be made in collaboration with R. Nairn to create various gadd mutants in knock-out experiments by targeted recombination using recombinant vectors. Studies will be continued on the expression of various DDI genes in mutant cells. The search for additional DDI genes will include studies with newly-isolated DNA repair genes such as the *XRCC1* gene isolated by L. Thompson.

Publications

Fornace AJ Jr, Nebert D, Hollander MC, Papathanasiou M, Fargnoli J, Holbrook N. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents, Molec Cell Biol 1989;9:4196-4203.

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Hentosh, P, Collins, ARS, Correll, L, Fornace, AJ Jr, Giaccia, A, and Waldren, CA. Genetic and biochemical characterization of the CHO-UV-1 mutant defective in postreplication recovery of DNA. Cancer Res 1990;50:2356-2362.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 07185-01 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of Radiosensitizers and Radioprotectors on DNA Damage Produced by X-rays

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

PI: A. J. Fornace, Jr. Senior Investigator LMPH NCI

COOPERATING UNITS *(if any)*

University of Wisconsin, Madison, WI (T. Kinsella).

LAB/BRANCH
Laboratory of Molecular Pharmacology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.02	.02	0

CHECK APPROPRIATE BOX(ES)

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

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

We have completed studies with the halogenated pyrimidine radiosensitizers, IUdR and BUdR. IUdR is currently in clinical trials as a radiosensitizer. This agent sensitizes cells to radiation after incorporation into cellular DNA in place of thymidine. We have previously shown that the yield of DNA strand breaks was increased in cells containing IUdR after x-irradiation. Current models of IUdR and BUdR radiolysis predict that only single strand damage should be produced in DNA. However, the lesion most important in x-ray lethality is probably DNA double strand breaks. We have found that radiolysis of IUdR in DNA of cells x-irradiated leads to mobile reactive intermediates which damage both the strand containing the IUdR and also the complementary strand which did not contain IUdR in these experiments. IUdR-induced strand breaks were almost as frequent in the unsubstituted strand as in the substituted strand. There was also a smaller increase in strand breaks in unsubstituted duplex DNA in cells containing IUdR-DNA. In cell survival experiments, cells undergoing only 1 doubling with IUdR showed almost as much radiosensitization as cells undergoing 2 doublings where both strands were substituted.

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

Project Description

Objective: To study the effect of radioprotectors and radiosensitizers on particular types of DNA damage.

Methods Employed

Alkaline and neutral elution. Standard cell culture techniques.

Major Findings

Radiolysis of DNA containing halogenated pyrimidines leads to the generation of mobile reactive intermediates which can produce double strand DNA damage, in particular double strand breaks.

Significance to Biomedical Research and the Program of the Institute

There are 2 major implications for radiotherapy:

1. Significant radiosensitization can be achieved in cells undergoing only 1 round of replication in halogenated pyrimidines. Therefore, efforts should be directed toward labelling as high a fraction of the tumor cells as possible, even if many have only been labelled on 1 DNA strand.

2. DNA double strand breaks are probably responsible for much of X-ray cell lethality. We infer from our studies that the use of maximum achievable concentrations of IUdR may be preferable, since it will produce the greatest density of intermolecular damage (double strand breaks) with radiation.

Proposed Course Terminate this year.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07186-01 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

DNA Damage by Alkylating Agents and Their Repair in Human Tumor Cells

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

PI:	A. J. Fornace, Jr.	Senior Investigator	LMPH NCI
	M. Papathanasiou (departure 7/1/90)	Visiting Fellow	LMPH NCI

COOPERATING UNITS *(if any)*

Applied Genetics, Freeport, New York (D. Yarosh).

LAB/BRANCH

Laboratory of Molecular Pharmacology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	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.)*

D. Yarosh, R. S. Day, III, and others have shown that approximately 20% of human tumor lines and viral transformed lines are hypersensitive to alkylating agents due to an apparent absence of O6-alkylguanine DNA alkyltransferase (O6AT); this phenotype has been designated mer$^-$. This enzyme removes alkylation damage at the 0-6 position of guanine but not at other sites in DNA. Dr. D. Yarosh in collaboration with our unit has been able to partially purify the enzyme from human liver and raise polyclonal antibodies to this protein. Studies have been initiated to develop monoclonal antibodies to this protein. Recently, a cDNA clone for a human O6AT has been isolated on the basis that it protects O6AT-deficient bacteria from certain alkylating agents (Tano, K, Shiota, S, Collier, J, Foote, RS, and Mitra, S. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O6-alkylguanine. Proc. Natl. Acad. Sci. USA 1990;87:686-690). Using oligonucleotides based on this sequence, we have isolated a full-length O6AT cDNA clone from a human liver library which we had constructed. Using this clone as a probe, we have found that O6AT mRNA was markedly reduced in all the mer$^-$ tumor cells lines (8) examined. The level of O6AT mRNA varied by 10-fold in mer$^+$ cell lines and correlated with known levels of the protein in particular cells. Our findings may have important implications in cancer therapy where agents such as BCNU are used. D. Yarosh has preliminary evidence that some brain tumors have no detectable O6AT activity. Determining which tumors are mer$^-$ and which mer$^+$ tumors have low levels of O6AT mRNA and protein would probably be useful in planning chemotherapy. Efforts are underway to develop approaches to measure O6AT mRNA and protein levels *in vivo*.

Project Description

Objective: To develop approaches to measure O6AT mRNA and protein levels *in vivo*, and to study the expression of this gene various human cell lines.

Methods Employed

Standard molecular biology and protein biochemistry approaches.

Major Findings

Polyclonal antibodies to this protein have been isolated, and efforts are directed at isolating high affinity polyclonal and monoclonal antibodies. O6AT expression vectors have been constructed to produce large amounts of this protein in bacteria; this material will be used to raise high affinity monoclonal antibodies and to further study the protein.

A full-length cDNA clone for human O6AT has been isolated. Using this cDNA as a probe, the level of O6AT mRNA has been measured by northern blots and by quantitative dot-blot hybridization. This mRNA was not detected in mer^- tumor cell lines and was reduced at least 100-fold compared to the level in mer^+ cells. The levels of this transcript in mer^+ cells varied by at least 10-fold.

Significance to Biomedical Research and the Program of the Institute

An understanding of this defect which occurs in approximately 20% of all human tumor lines would have obvious importance in both cancer treatment and carcinogenesis. Determining which tumors are mer^- and which mer^+ tumors have low levels of O6AT mRNA and protein would probably be useful in planning chemotherapy.

Proposed Course

Efforts are underway to measure the level of O6AT mRNA in tumor biopsy specimens. S1 nuclease protection analysis and PCR technology will be used to measure this transcript in clinical samples containing limited amounts of tissue. *In situ* hybridization will be developed by D. Yarosh to examine histologic sections. Efforts are underway to develop high affinity antibodies.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07187-01 LMPH

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Increased Expression of Stress-Induced Genes in Chemoresistant Tumor Cells

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

PI:	A. J. Fornace, Jr.	Senior Investigator	LMPH NCI
Others:	M. C. Hollander	Microbiologist	LMPH NCI
	M. Papathanasiou (departure 7/1/90)	Visiting Fellow	LMPH NCI

COOPERATING UNITS *(if any)*

Univ. of Chicago (R.Weichselbaum); Fox Chase Cancer Center, Philadelphia, PA (T. Hamilton); Smith, Kline, & French Laboratories, Philadelphia, PA (K. B. Tan); University of Berkeley, CA (R. Goth-Goldstein).

LAB/BRANCH

Laboratory of Molecular Pharmacology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	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.)*

A major problem in cancer chemotherapy is the emergence of drug-resistant cells. Chemotherapy-resistance is probably due to multiple mechanisms including altered uptake/excretion of drug, increased inactivation of drug, and altered host response such as increased DNA repair. As outlined in project number Z01 CM 06380-03 RO, we have isolated a variety of mammalian cDNA clones which code for transcripts specifically induced by DNA damage. We have initiated studies with a variety of human tumor cell lines which have been selected for resistance to cis-Pt diamminedichloride (DDP), nitrogen mustard (HN2), or other alkylating agents. Our initial studies have involved determining the level of different stress-induced transcripts in these chemoresistant tumor cell lines compared to their parent cell lines with normal sensitivity. Several examples of over-expression of certain of our DDI transcripts in DDP and alkylating agent resistant cells have been found. For example, in the case of *gadd33* RNA, this transcript was constitutively elevated in both DDP, melphalan, and HN2 resistant human tumor cell lines and also in a MNNG resistant Chinese hamster cell line. DDIA18 mRNA was found to be substantially elevated in certain chemoresistant human ovarian cell lines. These studies raise the important possibility that over-expression of certain DNA-damage-inducible genes may play a role in chemotherapy resistance.

Project Description

Objective: To determine the expression of DNA-damage-inducible transcripts in chemotherapy resistant tumor cells, and ultimately their role in chemotherapy resistance.

Methods Employed

Standard molecular biology techniques.

Major Findings

Several of our *DDI* transcripts have been found to be elevated in chemoresistant tumor cell lines including metallothionein II, DDIA18, and *gadd33*. It was interesting that DDIA18 mRNA was clearly elevated in several DDP-resistant ovarian cell lines. We have found that the level of this mRNA was unaffected by cell growth or cell cycle, and that the level of this mRNA was fairly constant in a variety of different cell lines. DDIA18 mRNA was only induced by DNA-damaging agents whose damage is repaired by nucleotide excision repair. The important lesions in DNA induced by DDP (interstrand crosslinks and intrastrand crosslinks) are removed by nucleotide excision repair.

Significance to Biomedical Research and the Program of the Institute

Identification of genes and regulatory pathways involved in certain forms of chemotherapy resistance may lead to better understanding of this serious clinical problem, and may ultimately provide insights into overcoming this problem.

Proposed Course

The short-term approach will be to determine DDI transcript levels in a variety of chemotherapy resistant cells and attempt to correlate this with cellular resistance. The long-term course involves a thorough characterization of these genes and their regulation.

Publications

Schilder, RJ, Hall, L, Monks, A, Handel, LM, Fornace, AJ Jr, Ozols, RF, Fojo, AT, Hamilton, TC. Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. Int J Cancer 1990;45:416-422.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07188-01 LMPH

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation and cDNA Cloning of DNA Polymerase ß in Chinese Hamster Cells

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

PI:	A. J. Fornace, Jr.	Senior Investigator	LMPH NCI
Others:	I. Alamo	Microbiologist	LMPH NCI
	M. C. Hollander	Microbiologist	LMPH NCI

COOPERATING UNITS *(if any)*

NCI (S. Wilson).

LAB/BRANCH
Laboratory of Molecular Pharmacology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

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.)*

ß-polymerase is one of the few mammalian DNA repair genes which have been isolated. This enzyme is responsible for the repair polymerase step ("gap filling" step) of DNA repair. In the case of short patch repair after damage by base damaging agents such as alkylating agents, ß-polymerase is the only polymerase utilized. The genes of repair polymerases in E. coli and yeast have been found to be DNA-damage inducible. In collaboration with S. Wilson who has cloned both human and rat ß-polymerase cDNA, we have found that ß-polymerase RNA is rapidly induced in Chinese hamster ovary (CHO) cells after exposure of the cells to alkylating agents or hydrogen peroxide. Induction did not occur after UV radiation, heat shock, perturbation of cell cycle, or exposure to other DNA damaging agents which did not induce high levels of adducts to single bases in DNA. This is the first demonstration of the induction of a DNA repair gene in higher eukaryotic cells specifically by DNA damage. In order to further elucidate the regulation of ß-polymerase in CHO cells, we have isolated the ß-polymerase cDNA clone from a CHO cDNA library. Sequence analysis demonstrated that ß-polymerase has been highly conserved in the Chinese hamster, rat, and human species. In collaboration with S. Wilson, we have found evidence that a DNA-damage-inducible trans-acting protein binds to the ß-polymerase promoter, and can lead to increased transcription of this gene. Recently, we have found evidence that the regulation of the ß-polymerase may involve one or more cellular kinases.

PHS 6040 (Rev. 1/84) GPO 914-9

Project Description

Objective: To study the regulation of ß-polymerase gene in mammalian cells.

Methods Employed

Standard molecular biology approaches.

Major Findings

The Chinese hamster ß-polymerase gene is the first mammalian DNA repair which has been found to be specifically induced by DNA-damaging agents. We have cloned and sequenced a Chinese hamster cDNA clone for ß-polymerase. Evidence has been found for a DNA-damage responsive element in the promoter of the ß-polymerase gene. Protein kinase inhibitors have been found to rapidly decrease the cellular level of this transcript.

Significance to Biomedical Research and the Program of the Institute

ß-polymerase plays an important role in the repair of damage by DNA damaging agents such as alkylating agents. Characterization of this gene and its regulation will probably provide insight into the response of mammalian cells to agents with relevance to both cancer therapy and carcinogenesis.

Proposed Course

See summary of work.

Publications

None

ANNUAL REPORT OF THE LABORATORY OF BIOLOGICAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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. The LBC's approach to achieving its mission is a balanced one with five objectives: (1) To advance the basic biochemistry/biology of the targets of interest; (2) to apply the techniques developed in molecular biology to advance drug discovery; (3) to collaborate with organic chemists in the development of agents to interfere with the targets of interest; (4) to study the pharmacology of new agents identified by the new DTP drug screen; and (5) to devise methods to improve the clinical effectiveness of anticancer agents.

Approximately 80% of the LBC's resources are 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 and protein modification reactions that are critical to the biological activity of specific cellular proteins. Specifically, the LBC focused on the biochemistry/biology of ADP-ribosylation factor (a small GTP-binding protein) and its role in protein secretion, the mitogen-induced activation of the synthesis of oligosaccharide moieties of extracellular matrix components, and on three cellular protein modification reactions: myristoylation, retinoylation, and isoprenylation. The remaining 20% of the LBC's resources are applied to the study of either traditional targets or active compounds with traditional or unknown mechanisms of action. In this regard, the Laboratory continued three projects to improve the therapeutic effectiveness of traditional antitumor agents. These include studies of the biochemistry and pharmacology of antipyrimidine antitumor agents and their targets, studies of the biochemistry of multidrug resistance, and investigations to improve the clinical utility of inducers of cellular differentiation. The results of these studies are summarized in the paragraphs that follow.

Protein modification reactions may determine the intracellular location and proper functioning of key proteins. Thus it may be possible to alter the activity of oncogene products or other important proteins by interfering with its localization in the plasma membrane or other cellular compartments. We are investigating the post-translational protein acylation with myristic acid (i.e. N-myristoylation), isoprenoids (i.e. isoprenylation) and retinoic acid (i.e. retinoylation) as potential new targets for chemotherapeutic drug development. Both myristoylation and isoprenylation have been shown to play roles in the targeting of cytoplasmic _onc_-kinases or retroviral _gag_ structural proteins to the inner plasma membrane cell surface. In the case of N-myristoylation, site directed mutagenesis designed to block N-myristoylation prevents membrane binding and inhibits both cellular transformation and viral replication. We have purified N-myristoyl transferase (NMT) from cow brain and shown that the

active enzyme is a 120 Kda dimer made up of a mixture of 4-5 different charge and size isoenzymes. A 52 Kda NMT isoenzyme subunit has been purified sufficiently for microsequencing and antibody production which will aid in the development of probes for screening for the NMT gene in cow brain cDNA libraries. Several new compounds have been identified as in vitro NMT inhibitors: the acyl CoA analog, S-(3-epoxymethylene)-dodecanoyl)-CoA, the acyl analog, 1-bromo-2-pentadecanone, and the multisubstrate analog, N-(S-(2-tetradecanoyl)-CoA)glycinamide. We have also found that N-myristoyl tetrazole inhibits N-myristoyl Coenzyme A synthetase both in vitro and in vivo. Both enzymes are important in the N-myristoylation pathway. We have also prepared cDNAs of the HIV p17gag and p27nef genes which encode N-myristoylated proteins. Bacterial and mammalian vectors of each cDNA have been constructed and used to express the gene proteins in E. coli and cos cells. The p17gag was purified from E. coli, N-myristoylated in vitro with cow brain NMT, and is now being used as a probe for identifying specific N-myristoyl-gag membrane "acceptors". The expressed nef has been shown to be localized in cos cells to discrete cytoplasmic structures rather than the plasma membrane.

Isoprenoids derived from mevalonic acid are required for a variety of cell biological functions including cell proliferation, membrane structure and function, cell adhesion and cytoskeletal functions. A subset of cellular proteins (including members of the ras oncogene- and G-protein families) is modified by covalent attachment of long chain isoprenoids (farnesyl, geranylgeranyl) to cysteines located in CAAX boxes close to the carboxyterminus. The functions of isoprenylation are not well understood. Farnesylation is thought to serve as a signal for other covalent modifications of ras-like proteins and nuclear lamin B, which increase their hydrophobicity and facilitate interaction of these proteins with membranes. Moreover , farnesylation is obligatory for the transforming activity of ras oncogenes. We have prepared the cDNA of a yeast gene which has been shown to be essential for the post-translational maturation of p21ras and for its subsequent membrane binding and transforming activity. We expressed this gene in E. coli and are testing its possible identity as an S-farnesyl transferase and thus as another potential target for anti-neoplastic drug development. At the cellular level, we are focusing on the role of isoprenylation in epithelial cells. We are using MDCK and other renal adenocarcinoma cell lines to investigate the requirement for isoprenylation in the regulation of cell shape and adhesion. We are developing permeabilized cell models capable of incorporating substrates and inhibitors of isoprenylation that are poorly taken up by intact cells. We found that renal adenocarcinoma cells incorporate 3H-mevalonate into proteins ranging in MW from greater than 130 Kd to 17 Kd. Cell lines differ in their mevalonate requirements for growth and viability, but in all cells examined, depletion of cellular mevalonate results in changes in cell-cell adhesive contacts and in tubulin organization. The underlying biochemical mechanisms are being investigated. We have developed methods to resolve isoprenoids from other cellular lipids and to assay protein isoprenyltransferase activity in cell free extracts. Initial studies on cell permeabilization suggest a novel role for ATP in cell adhesion, which is currently under investigation.

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) study the

metabolism of retinoic acid (RA), a potent inducer of differentiation; and b) 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. 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 methonolic-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 differentiation of HL60 cells.

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. The ARFs are a family of 21 kDa GTP-binding regulatory proteins, originally described and purified based on their ability to serve as the protein cofactor required in the in vitro ADP-ribosylation of Gs by cholera toxin. Cloning and sequence analyses of numerous ARF proteins as well as other small (20-25 Kda) GTP-binding proteins has resulted in the description of two structurally distinct families of low molecular weight GTP binding proteins, referred to as the "ras-related" and ARF families. The former is currently comprised of at least thirty distinct gene products. Of all the smaller GTP binding proteins only ARF has a defined biochemical activity, independent of GTP binding or hydrolysis. This activity has allowed the recent delineation of the ARF family into the bona fide ARF proteins and the structurally conserved ARF-related proteins. Recent results indicate that humans express at least three distinct ARF proteins with conserved activities both in vivo and in vitro. One of these human ARF genes, human ARF2, was cloned in a library screen of cDNAs which selected for inserts capable of stimulating the secretion of bFGF. These results are consistent with the observations that ARF is highly concentrated in mammalian cells to the Golgi complex and disruption of ARF1 in yeast results in a secretion defect. The role of ARF in the regulation of growth factor or other protein secretion is being very actively pursued. Specific functional domains of ARF proteins are being mapped with both point and deletion mutations as well as antibody probes. This work has identified a specific region of the protein which is not involved in nucleotide binding but which is critical to ARF function. This has allowed the construction of peptide inhibitors of ARF which are currently being used in in vitro assays of the role of ARF in protein secretion. Such results and analyses will likely provide new insights into the fundamental processes of protein secretion in higher eukaryotes as well as providing possible new targets for chemotherapeutic intervention in the specific case of the secretion of protein growth factors.

Elucidation of the sequence of events between growth factor-receptor interaction and the activation of obligatory pathways will yield an increasing

number of targets for drug modulation. Activation of uridine uptake is an early event in the mitogenic response, occurring within minutes of mitogenic stimulation of quiescent 3T3 mouse fibroblasts, compared with DNA synthesis which occurs 10-15 hours later. Data from our Laboratory indicate that the early activation of pyrimidine nucleotide synthesis is linked to the synthesis of components of the extracellular matrix (specifically, hyaluronate). Hyaluronate has been implicated as a factor involved in tumor invasion and metastasis. Thus elucidation of cellular regulatory mechanisms that control the synthesis of oligosaccharide moieties of extracellular matrix components could be useful to the discovery of agents with anti-invasive and anti-metastatic properties. Chromatographic analysis of the nucleotide pool in 3T3 fibroblasts 30 minutes after serum stimulation revealed an increase in UDP-glucuronide that corresponded to the increase in uridine uptake with regard to time course and magnitude. Other factors capable of stimulating uridine uptake, including EGF, PDGF, IL-1, and phorbol ester also caused an increase in UDP-glucuronide. Certain growth factors and various combinations of growth factors stimulated 3T3 fibroblasts to secrete hyaluronate. Of particular interest was the finding that conditioned media from a BT-20 human breast carcinoma (whose oncogene product is PDGF) stimulates hyaluronate synthesis by fibroblasts indicating intercellular communication between tumor and normal fibroblasts in the synthesis of hyaluronate induced by this human tumor. Studies into the biochemical mechanism for mitogen-stimulation of hyaluronate synthesis indicate that UDP-glucose dehydrogenase activity is increased following mitogen-stimulation of quiescent fibroblasts. This stimulation is an early event following mitogenic activation. Current studies are focusing on the regulatory role of UDP-xylose in the synthesis of proteoglycans and glycosaminoglycans. UDP-xylose is a potent inhibitor of UDP-glucose dehydrogenase and therefore may play an important role in determining the composition of the extracellular matrix. A GC/MS technique was developed for UDP-xylose as a prelude to study the synthesis and regulatory functions of UDP-xylose.

The relative contribution of de novo and salvage synthesis to tissue pyrimidine nucleotide pools is an important parameter in the rational design of anti-pyrimidine therapies, but was not previously measured in vivo because of the lack of suitable methodologies. Using stable isotopes, GC/MS methodology, and a novel method of data analysis, we measured de novo synthesis in mouse tumors and tissues. These values were compared with those for salvage determined with radiolabelled uridine. In a panel of mouse tumors, the contribution of de novo synthesis was at least four times the contribution of salvage synthesis. For normal tissues: de novo was more important in intestine, salvage more important in kidney, and the liver was dependent equally on de novo and salvage. Previous studies by our group demonstrated that an excess of ammonia can increase pyrimidine synthesis in liver and intestine because of the presence of mitochondrial carbamyl phosphate synthetase I (an enzyme lacking in most tumors). Host toxicity to PALA was reduced by providing excess ammonia by increasing dietary protein. Thus, it may be possible to improve the selectivity and therapeutic effectiveness of antipyrimidines through diet manipulation.

Studies on the cellular pharmacology of DUP-785, a new antipyrimidine antitumor agent showed that there is a direct correlation between inhibition of de novo pyrimidine synthesis, changes in pyrimidine nucleotide concentrations, and cell proliferation following short (<24 h) drug exposures; however, with prolonged

exposures (>24 h), there is a departure from this correlation in that restoration of pyrimidine nucleotide pools and de novo pathway activity does not restore cell proliferation. This information should be useful to the design of clinical protocols with this agent.

Amplified membrane glycoproteins (P-glycoprotein) have been identified in several cell lines exhibiting multidrug resistance (mdr). Studies were carried out to characterize P-glycoprotein phosphorylation and the associated Ca++ and phospholipid-dependent protein kinase C (PKC) activities in multidrug resistant cells. The overexpression of PKC is closely associated with the mdr phenotype in both leukemic and breast carcinoma cell lines. Our experiments showed that PKC activity is highly elevated in adriamycin-resistant HL60 cells in comparison to the parental cell line and PKC-gamma isoform is present in HL60/AR cells but not in wild type cells. It was also noted that HL60/AR cells and not the parental cells phosphorylate vinculin in vitro. These data suggest that PKC-gamma or its catalytic fragment is responsible for vinculin phosphorylation. A second part of this project is to study the role of protein kinase C in MCF-7/adriamycin-resistant cells and BC19 cells which were transfected with the mdr gene. We demonstrated that when BC19 cells are transfected with the protein kinase C gene, resistance to adriamycin is increased several-fold; increased resistance is associated with enhanced phosphorylation of P-glycoprotein and decreased drug accumulation. These results suggest that phosphorylation of P-glycoprotein may serve as an important pharmacological target for reversing the multidrug resistant process; protein kinase C may modulate the level of resistance to some anticancer drugs.

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

ZO1 CM 06163-06 LBC

PERIOD COVERED
October 1, 1989 to September 30, 1990

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
	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:
3.0	1.5	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.)*

The relative contribution of de novo and salvage synthesis to tissue pyrimidine nucleotide pools is an important parameter in the rational design of anti-pyrimidine therapies, but was not previously measured in vivo because of the lack of suitable methodologies. Using stable isotopes, GC/MS methodology, and a novel method of data analysis, we measured de novo synthesis in mouse tumors and tissues. These values were compared with those for salvage determined with radiolabelled uridine. In a panel of mouse tumors, the contribution of de novo synthesis was at least four times the contribution of salvage synthesis. For normal tissues; de novo was more important in intestine, salvage more important in kidney, and the liver was dependent equally on de novo and salvage.

Previous studies by our group demonstrated that an excess of ammonia can increase pyrimidine synthesis in liver and intestine because of the presence of mitochondrial carbamyl phosphate synthetase I (an enzyme lacking in most tumors). Host toxicity to PALA was reduced by providing excess ammonia by increasing dietary protein. Thus, it may be possible to improve the selectivity and therapeutic effectiveness of antipyrimidines through diet manipulation. Murine tumors made resistant to 5-fluorouridine were cross-resistant to cyclopentenyl-cytosine. The mechanism is related to the over-production of CTP, possibly through a change in CTP-synthase.

Studies on the cellular pharmacology of DUP-785 showed that there is a direct correlation between inhibition of de novo pyrimidine synthesis, changes in pyrimidine nucleotide concentrations, and cell proliferation following short (<24 h) drug exposures; however, with prolonged exposures (>24 h), there is a departure from this correlation in that restoration of pyrimidine nucleotide pools and de novo pathway activity does not restore cell proliferation. This information should be useful to the design of clinical protocols with this agent.

Objectives:

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; and to apply information gained from these studies to the treatment of cancer patients.

Major Findings:

Quantitation of pyrimidine synthesis de novo in the intact animal: The relative contribution of de novo and salvage synthesis to tissue pyrimidine nucleotide pools is an important parameter in the rational design of antipyrimidine therapies, but has not been measured in vivo because of the lack of suitable methodology. We measured the contribution of de novo synthesis to the total acid soluble uracil nucleotide pool in mouse tissues by analysis of the incorporation of label after i.p. infusion of L-[^{15}N]alanine. The contribution of salvage synthesis was measured by incorporation of label after i.v. infusion of [^{14}C]uridine. The results show that de novo synthesis makes the larger contribution to the intestine uracil nucleotide pool, salvage synthesis makes the larger contribution to the kidney pool, and de novo and salvage synthesis make approximately equal contribution to the liver pool. In the tumors studied (L1210, P388, B16, Nettesheim) the contribution of de novo synthesis was at least four times the contribution of salvage synthesis. Thus, these mouse tumors depend on de novo pyrimidine synthesis to a greater extent than normal mouse tissues. Current studies are attempting to exploit this difference for therapeutic benefit.

Effect of diet on the selective toxicity of antipyrimidines:

Previous work in our lab has shown that an excess of ammonia can cause increased pyrimidine synthesis in intestine as well as liver. The generally accepted mechanism for the stimulation of pyrimidine synthesis by ammonia is that overproduction of carbamyl phosphate by the mitochondrial enzyme carbamyl phosphate synthetase (CPS-I) results in leakage of carbamyl phosphate into the cytosol. This provides a source of carbamyl phosphate for pyrimidine synthesis that is not under regulatory control. The fact that tumor cells lack CPS-I activity has led us to investigate the possibility that the action of excess ammonia could provide a means for selectively protecting liver and intestine from the toxic effects of anti-pyrimidines. In early studies we are providing excess ammonia by increasing the protein content of the diet. We have found that animals on a 50% protein diet were much less sensitive (78% survival) to PALA (600 mg/kg, daily x4) compared to animals on an 18% protein diet (27% survival). The increased pyrimidine synthesis caused by excess ammonia also results in increased levels of orotate, which could inhibit activation of 5FU by competing with orotate phosphoribosyl transferase. Increasing dietary protein has been reported to decrease host toxicity of 5FU with no decrease in antitumor activity. Future studies will concentrate on defining the response of normal tissues and tumors to PALA and 5FU with animals on different dietary protein levels. This information will be used to design a PALA, 5FU, high protein diet combination.

Uridine salvage-deficient mutants: An alternative approach to the use of inhibitors of uridine salvage to determine the role of salvage in tumor resistance is the development of mutants lacking salvage capability. We used 5-fluorouridine to select for cells that lack uridine kinase. Two mutant colonies each of P388 and L1210 cells were isolated. These colonies were cross-resistant to cyclopentenyl cytosine and failed to incorporate labeled uridine. However, these colonies contained normal levels of uridine kinase that behaved as the wild type enzyme with regard to allosteric control mechanisms. HPLC analysis demonstrated elevated CTP concentrations which could account for the selected phenotype. Current studies are investigating CTP-synthase as the altered enzyme responsible for the phenotypic change.

Cellular pharmacology of DUP-785, a new anticancer agent: DUP-785 is an inhibitor of dihydroorotate dehydrogenase currently undergoing clinical evaluation for anticancer activity. We developed a GC/MS method to quantitate dihydroorotate that accumulates in cultures of L1210 cells exposed to growth inhibitory concentrations of DUP-785. This method was used to follow the onset, extent, and duration of inhibition of de novo pyrimidine synthesis in intact L1210 cells and to compare this inhibition with cellular concentrations of pyrimidine nucleotides and cell proliferation. The dose-dependent increase in the rate of dihydroorotate accumulation followed closely the dose-dependent inhibition of cell proliferation. Inhibition of de novo pyrimidine synthesis is rapid causing decreased UTP and CTP pools by 4 hr and a corresponding effect on dihydroorotate accumulation which reached a constant maximum rate by 24 hr that continued for up to 7 days with continuous drug exposure. Drug removal resulted in rapid restoration of pathway activity and cellular pools of UTP and CTP in cells exposed for short (<24 hr) or long (>24 hr) periods of time.

There was a direct correlation between inhibition of de novo pyrimidine synthesis, changes in pyrimidine nucleotide concentrations, and cell proliferation following short (<24 hr) drug exposures; however, with prolonged exposures (>24 hr) there is a departure from this correlation in that restoration of pyrimidine nucleotide pools and de novo pyrimidine pathway activity does not restore cell proliferation. During long term exposures, dihydroorotate excretion rates were constant (0.83 nmole/hr/10 cells) for up to 8 days even though <0.1% of the cells were capable of cell proliferation. This maximum cell kill did not increase with drug exposures up to 96 hr. In contrast to short exposures, drug removal or uridine addition did not restore cell proliferation to cultures exposed for >24 hr; regrowth of the surviving fraction (ie. the <0.1%) occurs with drug removal. Current studies will determine if other inhibitors of de novo synthesis (PALA, pyrazofurin) behave as DUP-785 and whether the observed effects are unique to L1210 cells.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06167-06 LBC

PERIOD COVERED

October 1, 1989 to September 30. 1990

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

Inhib. of Lipoprotein-Dependent Cell Transformation & Retroviral Replication

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

PI:	R.L. Felsted	Research Chemist	LBC, NCI
Others:	C. Glover	Microbiologist	LBC, NCI
	K. Hartman	Chemist	LBC, NCI
	B. Burnette	IRTA	LBC, NCI
	G. Yu	Visiting Associate	LBC, NCI
	S. Bouziane	Visiting 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:
5	5	.2

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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.)*

We are investigating the post-translational protein acylation with myristic acid (i.e. N-myristoylation) and protein modification with isoprenoids (i.e. S-isoprenylation) as potential new targets for chemotherapeutic drug development. Both phenomenon have been shown to play roles in the targeting of cytoplasmic onc-kinases or retroviral gag structural proteins to the inner plasma membrane surface. In the case of N-myristoylation, site directed mutagenesis designed to block N-myristoylation prevents membrane binding and inhibits both cellular transformation and viral replication, respectively. We have purified N-myristoyl transferase (NMT) from cow brain and shown that the active enzyme is a 120 Kda dimmer made up of a mixture of 4-5 different charge and size isoenzymes. A 52 Kda NMT isoenzyme subunit has been purified sufficiently for microsequencing and antibody production which will aid in the development of probes for screening for the NMT gene in cow brain cDNA libraries. Several new compounds have been identified as in vitro NMT inhibitors: the acyl CoA analog, S-(3-epoxymethylene)-dodecanoyl)-CoA, the acyl analog, 1-bromo-2-pentadecanone, and the multisubstrate analog, N-(S-)(2-tetradecanoyl)-CoA)glycinamide. We have also found that N-myristoyl tetrazole inhibits N-myristoyl Coenzyme A synthetase in vitro as well as HIV replication in vivo. Both enzymes are important in the N-myristoylation pathway. We have also prepared cDNAs of the HIV p17gag and p27nef genes which encode for N-myristoylated proteins. Bacterial and mammalian vectors of each cDNA have been constructed and used to express the gene proteins in E. coli, N-myristoylated in vitro with cow brain NMT, and are now being used as probes for identifying specific N-myristoyl-protein membrane "acceptors". The expressed nef has been shown to be localized in cos cells to discrete cytoplasmic structures rather than the plasma membrane. In the case of S-isoprenylation, we have prepared the cDNA of a yeast RAM gene, expressed it in E. coli, and are testing its possible identity the S-farnesyl transferase required for p21ras transforming activity.

Objectives

In this project we will examine the role of post-translational lipid modification on onc-tyrosine kinases and retroviral gag structural proteins as a target for the chemotherapeutic inhibition of cellular transformation and viral reproduction. Specifically, we will study protein acylation with myristic acid (i.e. N-myristoylation) and protein modification with isoprenoids(i.e. S-isoprenylation). Specific goals include (1) studying the biochemistry and molecular genetics of N-myristoyl transferase (NMT); (2) developing inhibitors to myristoyl CoA synthetase and NMT; (3) characterizing the mechanism of cytoplasmic translocation of N-myristoylproteins; (4) characterizing the mechanism of N-myristoylprotein membrane binding, specifically with respect to the identification of HIV N-myristoylprotein membrane acceptors; and (5) studying the role of S-isoprenylation in the function and subcellular targeting of proteins.

Major Findings

1. High affinity binding of an N-terminal myristoylated p60src peptide. 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 in 50 mM NaCl caused an apparent inhibition of binding. However, no significant effect 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. Chemical characterization of p17gag from HIV as an N-terminally myristoylated protein. The N-terminal p17gag protein of HIV 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 p17gag was isolated by immunoprecipitation and subject to partial acid hydrolysis. [3H]Myristoylglycine generated by the hydrolysis was derivatized, to 4-(p-nitrobenzylidene)-2-tridecanoloxazol-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. Synthesis and characterization of N-myristoyl transferase inhibitors. A number of substrate and product analogs were synthesized and tested as in vitro

inhibitors of bovine brain N-myristoyl transferase. The acyl CoA analog, S-(2-ketopentadecyl)-CoA, completely inhibited NMT in the presence of 80 uM myristoyl CoA. Decreasing but significant inhibition was also observed with the acyl CoA analogs, S-(2-bromotetradecanoyl)-CoA and S-(3-epoxymethylene)-CoA, the myristoyl analog, 1-bromo-2-pentadecanone, and multisubstrate analog, N-(S-2-tetradecanoyl)-CoA glycinamide. Two acyl CoA analogs S-(cis-3-tetradecanoyl)-CoA and S-(3-tetradecynoyl)-CoA, apparently activate the NMT causing an increased formation of the N-myristoylpeptide product and in the case of the latter compound also resulting in an additional less hydrophobic N-acylpeptide product. The 1-bromo-2-pentadecanone exhibited an apparent non-competitive inhibition with respect to myristoyl CoA while the other four inhibitors displayed competitive kinetics (ki's = 0.1 to 19 uM) with respect to myristoyl CoA. The noncompetitive inhibitor, 1-bromo-2-pentadecanone, also inhibited NMT in an irreversible time dependent manor with k_{+1} = 2 x 10-4 min-1.

Proposed Course.

N-Myristoylation and S-isoprenylation are thought to be important cellular phenomenon. The fact that such unusual or rare lipids are utilized with such absolute specificity by transforming onc-kinases suggest they have a central role in the control of cellular growth and differentiation. Furthermore the involvement of N-myristoylation in mammalian retrovirus replication makes this phenomenon an attractive target for drug development directed at a number of potential diseases. The work in this project is designed to clarify several specific aspects concerning the process of N-myristoylation and S-isoprenylation. From these studies, it may be possible to design novel new compounds for specifically blocking these lipid modification pathways. These agents may then provide us with pharmacologic means for specifically treating N-myristoylation and/or S-isopenylation dependent cellular transformation and/or mammalian retroviral related disorders.

We propose to investigate four specific aspects of the N-myristoylation pathway which may be susceptible to direct chemotherapeutic manipulation. These include, (i) myristoyl CoA synthetase(s), (ii) NMT, (iii) cytoplasmic translocation of N-myristoylproteins, and (iv) of the binding of N-myristoylproteins to specific membrane receptors. We also propose to characterize the protein encoded by the yeast RAM gene which is known to be essential for p21ras membrane binding and to study S-isoprenylation of proteins in general. We would predict that these steps are essential to the overall N-myristoylation and S-isoprenylation pathways and therefore each represents an opportunity to block the resulting lipoprotein dependent transformation and/or viral replication.

(i) Myristoyl CoA Synthetase. An assay for myristoyl CoA synthetase has been developed and is being used to test possible inhibitors. Specifically identified enzyme inhibitors will be tested for their effect on protein N-myristoylation and HIV replication in tissue culture.

(ii) N-Myristoyl Transferase(s). The physical, chemical and kinetic properties of the purified bovine brain NMT will be characterization and compared with the previously characterized yeast enzyme. The purified enzyme will be used to

synthesize polynucleotide probes for screening cDNA libraries for the mammalian NMT gene.

(iii) N-Myristoylprotein Cytoplasmic Translocation. N-Myristoylprotein associations and other cytoplasmic proteins will be studied in order to evaluate the role of such soluble complexes in the translocation of the N-myristoylproteins from their site of synthesis in the cytoplasm to their specific membrane targets on the inner plasma membrane.

(iv) N-Myristoylprotein Membrane Binding. Membrane binding sites for the HIV p17gag and p27nef N-Myristoylproteins will be studied using bacterially expressed proteins as ligand probes. N-myristoylprotein receptors will be purified, characterized, and their genes cloned. Specific agents will be designed to block N-myristoylprotein receptor interaction. These will be tested as inhibitors of HIV replication in tissue cultured cells.

(v) S-Isoprenylation. In collaboration with Drs. Friedman and Kahn, the yeast ras maturation-dependent RAM gene will be expressed in E. coli and characterized. An in vitro S-isoprenylation assay will be developed in order to determine the possible RAM gene identity with the gene encoding S-fanesyl transferase. The expressed enzyme will be characterized and compared with the recently described mammalian enzyme. The general role of isoprenoids in protein function will be examined.

Publications.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06181-05 LBC

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The ARF Family as Regulators of Protein Secretion and Other Cellular Processes

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

PI:	Richard A. Kahn	Pharmacologist	LBC, NCI
Others:	Cherrie Rulka	Biologist	LBC, NCI
	Jenny Clark	Microbiologist	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:
3.0	3.0	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 ARFs are a family of 21 kDa GTP-binding regulatory proteins; originally described and purified based on their ability to serve as the protein cofactor required in the in vitro ADP-ribosylation of Gsα by cholera toxin. Cloning and sequence analyses of numerous ARF proteins as well as other small (20-25 Kda) GTP-binding proteins has resulted in the description of two structurally distinct families of low molecular weight GTP binding proteins; referred to as the "ras-related" and ARF families. The former is currently comprised of at least thirty distinct gene products. Of all the smaller GTP binding proteins only ARF has a defined biochemical activity, independent of GTP binding or hydrolysis. This activity has allowed the recent delineation of the ARF family into the bona fide ARF proteins and the structurally conserved ARF-related proteins. Recent results indicate that humans express at least three distinct ARF proteins with conserved activities both in vivo and in vitro. One of these human ARF genes, human ARF2, was cloned in a library screen of cDNAs which selected for inserts capable of stimulating the secretion of bFGF. These results are consistent with the observations that ARF is highly concentrated in mammalian cells to the Golgi complex and disruption of ARF1 in yeast results in a secretion defect. The role of ARF in the regulation of growth factor or other protein secretion is being very actively pursued.

Specific functional domains of ARF proteins are being mapped with both point and deletion mutations as well as antibody probes. This work has identified a specific region of the protein which is not involved in nucleotide binding but which is critical to ARF function. This has allowed the construction of peptide inhibitors of ARF which are currently being used in in vitro assays of the role of ARF in protein secretion.

Introduction

The fields of signal transduction and cellular regulation have largely been redefined by the recent discoveries of the central roles for regulatory GTP-binding proteins in a diverse array of cellular functions and signaling systems; including olfaction, taste, sight, activation and inhibition of adenylate cyclase, phospholipase C, and numerous ion channels, to name only a few. Molecular biology has aided in this revolution both by allowing the identification and sequencing of these families of proteins as well as providing the tools necessary to study their functions in a wide variety of cell types. Structural analyses of over 50 GTP binding proteins has resulted in the description of three sub-families of these regulatory proteins, as seen in figure 1.

The trimeric G-proteins each have an alpha subunit capable of binding GTP and in most cases serving as substrate for one of several bacterial toxins; cholera, pertussis, or botulinum C3 toxins. There are currently at least 16 known distinct alpha subunits. The most numerous sub-family is that of the "ras-related" proteins which currently numbers about 30 members and estimates range between 30 and 100 as to how many will eventually be found. These are all monomeric GTP-binding proteins with structural similarities to the oncogenic p21 ras proteins which extend beyond the consensus GTP-binding domains. The third sub-family is the ARF family with at least 6 members identified to date, including at least 3 bona fide ARF proteins and 3 ARF related proteins. These too are monomeric GTP-binding proteins but with structures intermediate between the two other sub-families and thus clearly distinguishable. While work in this project clearly focuses on the function and mechanism of ARF action, it is expected that whatever is learned about this class of GTP-binding proteins may well relate to the other structurally related members of the superfamily of regulatory GTP-binding proteins.

Objectives

The objectives of this project encompass biochemical, genetic and molecular biological approaches to the understanding of the role and molecular mechanism of ARF action in protein secretion and other biological processes as well as detailing the other components in the ARF pathway. Analysis of the functional domains of the ARF protein will be used both in the construction of inhibitors of ARF as well as in the design of conditional lethal alleles of ARF1 in yeast for the purpose of library screens to identify other proteins in the ARF pathway.

Major Findings

1) We have cloned and sequenced two human ARF CDNAS. One was obtained by cross-species hybridization with the bovine probe. The other, HARF2, was cloned from a cDNA expression vector in a screen which was designed to identify genes involved in the regulation of BFGF secretion. A role for this ARF protein in the regulation of BFGF secretion is suggested but remains unproven. We have constructed plasmids allowing the expression of human ARF genes in yeast and shown these to rescue lethality resulting from the deletion of both yeast ARF genes, thus demonstrating conservation of ARF function between yeast and man. This work on human ARFs is being submitted for publication in June, 1990.

2) Synthetic peptides were synthesized and injected into rabbits to generate mono-specific antibodies to ARF proteins. These proved to be good probes for fluorescent and electron immunolocalization of ARF in cells. Results indicate a high degree of concentration of ARF on the cytosolic surface of cis-Golgi membranes. In related studies we have investigated the effects of ARF mutations on secretion in yeast and found (a) incomplete glycosylation of invertase in the arf1-strain which is rescued by expression of ARF1 and (b) synthetic lethality resulting from the double mutants of arf1- with either sec7-1, sec21-1, or bet2-1. Thus, genetic data further suggests a role for ARF in protein secretion.

3) We have constructed 9 point mutations in yeast ARF1 and analyzed the consequences in vivo by expressing the mutant in yeast in an arf1-background, and in vitro by expressing the proteins in bacteria, purifying them to homogeneity and analyzing ARF dependent activities. Results demonstrate for the first time: (a) the requirement in vivo but not in vitro for the covalent attachment of myristic acid at the amino terminal glycine, (b) lack of requirement for systeine 156, the only cysteine in ARF, (c) lack of essential N-glycosylation at the sole consensus site, (d) the importance of aspartic acid 26 to both ARF activity and nucleotide affinity, (e) the role of glutamine 71 in nucleotide handling as the leucine mutant results in a dominant lethal phenotype in yeast, (f) the role of asparagine 126 in activation of ARF as this allele is a dominant loss of function mutant in yeast, and finally, (g) the role of tryptophan 66 in nucleotide handling, probably through coordination of the magnesium, as the W66L mutant turns out to be a temperature sensitive, recessive mutation. The last three mutants listed have been used or are being used to screen yeast libraries for suppressors of ARF-dependent activities. One such screen has been successful to date and numerous genes are being analyzed to define new genes and gene products in the ARF pathway. This work is not yet written up for publication.

4) Analysis of five different synthetic peptides, derived from different regions of the mammalian ARF proteins, has provided the first evidence of a specific domain critical to ARF function, distinct from those domains involved in nucleotide binding. This was supported by construction of a deletion mutation of human ARF1 which was shown to possess wild-type nucleotide handling properties but lack all ARF activities. The peptide corresponding to the part of the protein deleted was synthesized and shown to be a very potent inhibitor of ARF activity both in the ARF assay and in an in vitro protein secretion assay, demonstrating directly an obligate role for ARF in Golgi transit.

Significance

This laboratory has provided the first and only data indicating a physiological role for ARF proteins in eukaryotic cells. Results indicate a role in the regulation of secretion of growth factors (BFGF, KFGF) and other secreted proteins. Further, we have in hand both specific inhibitors of ARF activity that should prove invaluable in further analysis of protein secretion as well as clones of suppressors of ARF functions which may well prove to include several other components in the ARF/protein secretion pathway. Such results and analyses will likely provide new insights into the fundamental processes of protein secretion in higher eukaryotes as well as providing possible new targets for chemotherapeutic intervention in the specific case of the secretion of protein growth factors.

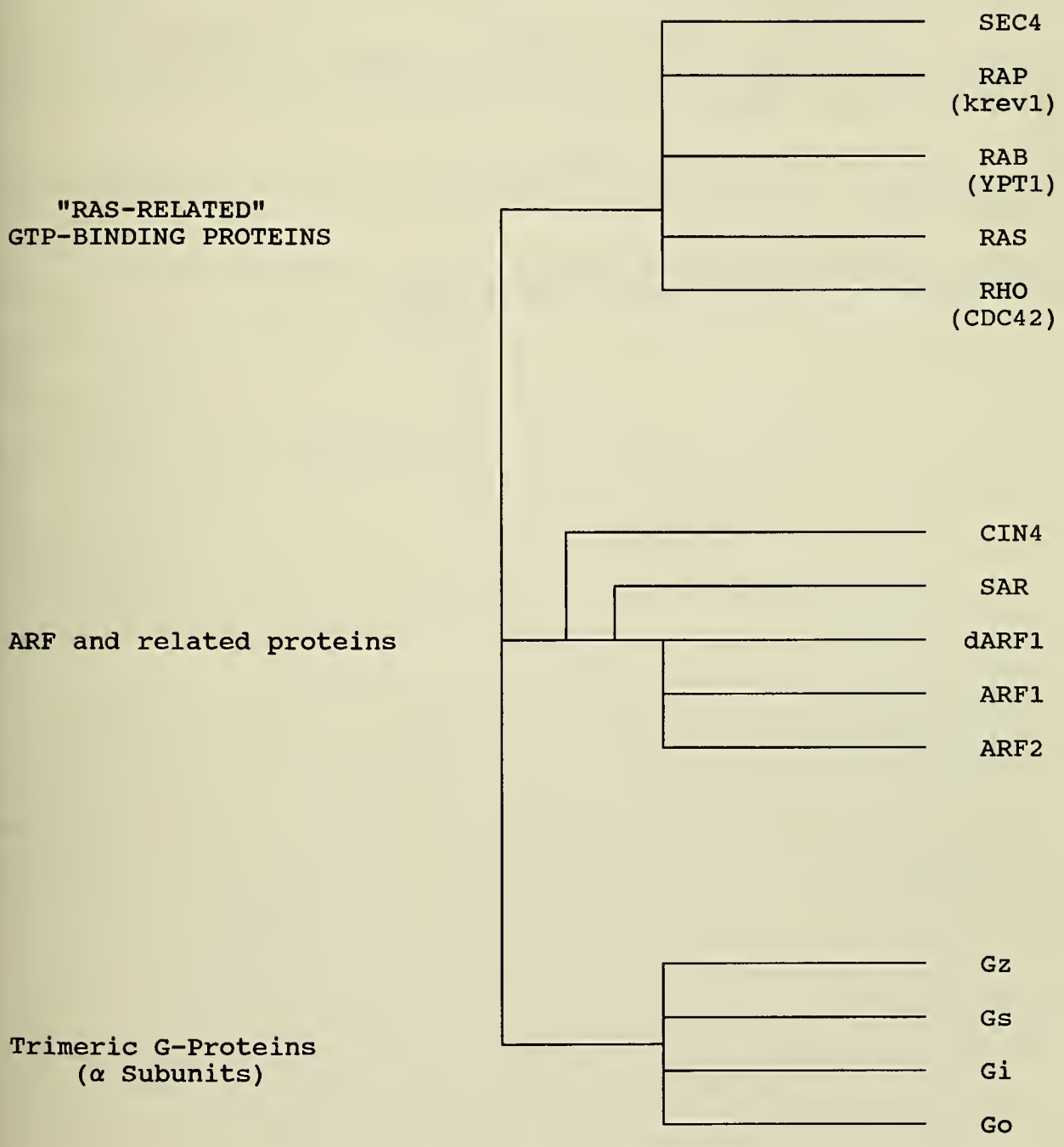

Figure 1. Sub-families of regulatory GTP-binding proteins.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06190-03 LBC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one 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:	Gang Yu	Visiting Associate	LBC NCI
Others:	Angelo Aquino	Visiting Fellow	LBC NCI
	Marian Johnson Thompson	IPA	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:
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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 purpose of this project is to characterize P-glycoprotein phosphorylation and the associated Ca^{++} and phospholipid-dependent protein kinase C (PKC) activities in multidrug resistant cells. The overexpression of PKC is closely associated with the mdr phenotype in both leukemic and breast carcinoma cell lines. Our experiments showed that PKC activity was highly elevated in adriamycin-resistant HL60 cells in comparison to the parental cell line and PKC-gamma isoform is present in HL60/AR cells but not in wild type cells. It was also noted that HL60/AR cells and not the parental cells phosphorylated vinculin in vitro. These data suggest that PKC-gamma or its catalytic fragment may have been responsible for vinculin phosphorylation. A second part of this project is to study the role of protein kinase C in MCF-7/adriamycin-resistant cells and BC19 cells which were transfected with the mdr gene. We have demonstrated that when BC19 cells are transfected with the protein kinase Cα gene, resistance to adriamycin is increased several-fold; increased resistance is associated with enhanced phosphorylation of P-glycoprotein and decreased drug accumulation. These results suggest that phosphorylation of P-glycoprotein may serve as an important pharmacological target for reversing the multidrug-resistant process; protein kinase C may modulate the level of resistance to some anticancer drugs.

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. In our previous investigation, we reported that PKC was more highly expressed in an Adriamycin-resistant variant of HL60 cells than in the parental cell line, and that the proteolysis of PKC appeared to be enhanced in drug-resistant cells. PKC is a family of enzymes with strikingly different responses to proteolysis and phorbol ester activation. The first part of this proposal is to determine the relative abundance of the three major isoforms of PKC in mdr cells in comparison to sensitive cells. This will be followed by introducing the certain isoform of PKC gene into BC19 cells, a clone of MCF-7 cells expressing a high level of the mdr gene but a very low level of PKC to determine the role of protein kinase C and P-glycoprotein phosphorylation in multidrug resistant cells.

Significance.

While P-glycoprotein over expression is a common if not invariable feature of mdr cell lines, several questions remain unanswered concerning mdr. Studies have revealed that P-glycoprotein is also phosphorylated. However studies of the posttranslational modification of P-glycoprotein have been limited. Changes in the phosphorylation of P-glycoprotein could alter the activity of this protein in different cells. In this regard, some studies have demonstrated that incubation of drug-sensitive and resistant MCF-7 human breast cancer cells in the presence of activators of protein kinase C decreased the sensitivities of both cell lines. This change was associated with alterations in the net intracellular accumulation of vincristine in both the sensitive and mdr cell lines. This implies that phosphorylation of P-glycoprotein by protein kinase C may regulate the efflux of drugs and other xenobiotics in mdr cells. But the direct association between phosphorylation of P-glycoprotein and the function of that protein remains to be established.

Major findings.

The isoform pattern of protein kinase C was examined in wild-type and Adriamycin-resistant (HL-60/AR) HL60 leukemia cells. Analyses were carried out by immunobloting with mouse monoclonal antibodies against PKC-α and PKC-ß and a rabbit polyclonal antibody against the variable region of PKC-γ. HL60/AR cells contained an equivalent level of PKC-α and a lower amount of PKC-ß than HL60 cells. In contrast, only HL60/AR cells contained PKC-γ. On the other hand, in MCF-7/Adriamycin-resistant cells the only isoform of PKC is α. No other isoform can be detected. These results indicate that the regulation of this family of isoenzymes is altered in drug-resistant cells.

A clone of MCF-7 cells, expressing high level of the mdr gene but very low levels of PKC, were transfected with a plasmid containing the cDNA for PKC-α under the control of the Rous Sarcoma virus promoter. Initial transfectants were screened by their resistance to G418 and assayed for PKC activity. Among the G418-resistant clones, clone 3 contained the highest cDNA copy number, as well as the highest level and activity of PKCα. Clone 3 and clone 15 were tested using a clonogenic assay for their resistance to Adriamycin after

continuous exposure to drug for 10 days or for 2 hr. Transfectant clone 3 and clone 15 increased resistance by 5-8 fold. Similar results were noted when cells were treated with vinblastine. Cytotoxicity was also determined in the presence of PKC activator, phorbol dibutyrate (PDBu). Following exposure to 200 nM PDBu for 2 hr, the IC_{50} for Adriamycin increased 6- and 11-fold for clone 15 and clone 3 cells, respectively. Drug retention in several of the PKC-α-transfected cell lines was determined. The retention of [^{3}H]vinblastine during a 2 hr exposure interval revealed that clones 15, 20 and 3 retained 1.8, 2.3 and 2.9-fold less drug than the parental cell line. These results suggest that a specific isoform of PKC is responsible for increased drug resistance and decreased drug retention.

Experimental Design and Methods.

Human promyelocytic cell line HL60 was obtained from the American Type Culture Collection. HL60/AR 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 PCF-7 cells transfected with the mdr-1 gene were provided by Dr. Kenneth H. Cowan, Medicine Branch, NCI.

The cDNA for PKCα was cloned into the Hind III site of plasmid pRSVneo expression vector. Lipofectin (Gibco/BRL) was used for transfection according to the instruction of the manufacturer. PKC assays and the immunoblotting and detection of PKC were performed as described previously. Phosphorylation assays were performed in the presence and absence of Ca^{2+} and phosphatidylserine to quantitate PKC-dependent phosphorylation. For analysis of phosphorylation *in vivo*, cells will be incubated with $H_3{}^{32}PO_4$ for 4 hr in medium.

Publications.

Aquino A, Warren BS, Omichinski J, Hartman KD, Glazer RI. Protein C-gamma is present in adriamycin resistand HL60 leukemia cells, Biochem Biophys Res Commun 1990;166:723-728.

Yu G, Aquino A, Fairchild CR, Cowan KH, Ohno S, Glazer RI. Adriamycin resistance in MCF-7 cells expressing P-glycoprotein following transfection with protein kinase Cα, Proceedings of the eighty-first annual meeting of the American Association for Cancer Research, 1990;Vol 31:366.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06196-02 LBC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulation and Function(s) of Isoprenylation in Cultured Epithelial Cells

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

PI:	Susan J. Friedman	Pharmacologist	LBC NCI
Others:	Peter Bogner	Visiting Fellow	LBC NCI
	Eduardo Sainz	Chemist	LBC NCI

COOPERATING UNITS *(if eny)*

None

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH. Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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.)*

Isoprenoids derived from mevalonic acid are required for a variety of cell biological functions including cell proliferation, membrane structure and function, cell adhesion and cytoskeletal functions. A subset of cellular proteins (including members of the ras oncogene- and G-protein families) is modified by covalent attachment of long chain isoprenoids (farnesyl, geranylgeranyl) to cysteines located in CAAX boxes close to the carboxyterminus. The functions of isoprenylation are not well understood. Farnesylation is thought to serve as a signal for other covalent modifications of ras-like proteins and nuclear lamin B, which increase their hydrophobicity and facilitate interaction of these proteins with membranes. Moreover, farnesylation is obligatory for the transforming activity of ras oncogenes. Much remains to be learned about isoprenylation in epithelial cells, which is the primary focus of this project. We are using MDCK and other renal adenocarcinoma cell lines to investigate the requirement for isoprenylation in the regulation of cell shape and adhesion. In addition, we are developing permeabilized cell models capable of incorporating substrates and inhibitors of isoprenylation that are poorly taken up by intact cells. We have found that renal adenocarcinoma cells incorporate 3H-mevalonate into proteins ranging in MW from greater than 130 Kd to 17 KD. Cell lines differ in their mevalonate requirements for growth and viability, but in all cells examined depletion of cellular mevalonate results in changes in cell-cell adhesive contacts and tubulin organization. The underlying biochemical mechanisms are being investigated. Eduardo Sainz , a chemist in my laboratory, has developed HPLC and TLC methods to separate isoprenoids from other cellular lipids and an assay for protein prenyltransferase activity in cell free extracts. Initial studies on cell permeabilization suggest a novel role for ATP in cell adhesion, which is currently under investigation.

Objectives.

Our objectives are (1) to investigate the role of isoprenylated proteins in the regulation of cell shape and cell adhesion and (2) to develop in vitro assays and permeabilized cell models for studies on protein isoprenylation.

Results.

a. Mevalonic acid requirements for growth and viability. Six cell lines (SN12K1, A-498, CAKI-1, RXF-393, U031 and MDCK were grown in sterol-containing medium in 24 well plates, blocked with mevinolin (a competitive inhibitor of HMG CoA reductase) in the presence of differing quantities of exogenous MVA and assayed after 72 hr by the SRB cytotoxicity assay. The level of MVA required to protect cells varied as much as 10-fold among the different cell lines, which presumably reflects differences in their ability to take up exogenous MVA. Based on these experiments, particular cell lines were selected for further study.

b. Incorporation of 3H-MVA into isoprenylated proteins. Starting with 100 mCi of 3H-sodium borohydride, approximately 40 mCi of high specific activity 3H-MVA can be synthesized from mevaldic acid precursor. Purity was approximately 90% as determined by TLC. Selected renal adenocarcinoma cell lines were labeled in the presence of mevinolin and 3H-MVA for 18 hrs, then extracted with organic solvents to remove noncovalently bound lipids. The pattern of labelling in each of the lines was similar by 1D-SDS gel electrophoresis. Discrete protein bands ranging in MW from greater than 130 KD to approximately 17 Kd were labelled. Most of the labelling was present in proteins with MW 20 Kd and below.

c. Cell shape and cytoskeletal changes in MDCK cells treated with mevinolin. Following treatment with mevinolin in the absence of exogenous MVA, there was a progressive transformation of the population from a flat cobblestone epithelioid morphology to one in which cell-cell junctions were altered (visualized with DIC optics). Cells became elongated and acquired long thin tubulin-containing processes (determined by immunofluorescence microscopy). This striking reorganization of tubulin was absent in cells treated with taxol or dolastatin analogs which affect microtubule functions. A characteristic feature of mevinolin-treated kidney cultures was the presence of numerous areas encircled by elongated cells resembling "domes" (regions of transepithelial fluid transport) in control cultures. The peripheries of these regions were heavily stained with Bodipy phallacidin, which reacts with actin microfilaments. After prolonged mevinolin treatment, these structures were the only visible cellular elements remaining. Their further characterization by electron microscopy is underway. Following drug washout, cell morphology and growth rate reverted to normal although the frequency of "dome-like" structures was markedly increased. The possibility that "dome" formation may be a renal cell stress response is suggested by a recent report on the induction of "domes" by glucose deprivation.

d. Biochemical studies on mevinolin effects on microtubules. To determine whether mevinolin's effects resulted from a direct interaction of the drug with tubulin, we examined the effects of the drug on the rate of in vitro

polymerization of tubulin. In the concentration range 20-100 uM, mevinolin exhibited "taxol-like" effects-i.e. it enhanced the rate of polymerization and slowed the rate of cold-induced depolymerization of calf-brain tubulin. The rapidity of this effect contrasts with the slow development of changes in tubulin organization in mevinolin-treated cells despite relatively efficient drug uptake. Taxol itself failed to produce mevinolin-like changes in tubulin organization in these cells. These results suggest that a direct effect of mevinolin on tubulin is not primarily responsible for the cellular changes observed. We have turned our attention to the possible involvement of isoprenylation in functions of microtubule associated proteins.

e. Development of in vitro assays for farnesyltransferase activity. This project is being carried out collaboratively with other labs in LBC (Dr. R. Felsted, Dr. R. Kahn). Reticulocyte lysate was used as a source of enzymes for converting MVA to farnesylpyrophosphate, and transferring farnesyl to p21 ras. The assay will be used to determine whether the product of the yeast RAM/DPR1 gene, known to be required for ras processing, is farnesyltransferase. We are beginning to develop permeabilized mammalian cell models for studying protein prenylation and developing/testing novel inhibitors of the pathway. The rationale for using permeabilized cells is the impermeability of intact cells to prenyltransferase substrates. Various permeabilization methods were tested using HL-60 and MDCK cells. HL-60 was effectively permeabilized with digitonin, alpha-toxin and ATP (assessed by lucifer yellow uptake and p-nitrophenylphosphate hydrolysis), whereas MDCK was permeabilized with digitonin but not alpha-toxin, and was detached by ATP. This latter finding is intriguing in view of the reported association of Na+/K+ATPase and integrins in cultured renal and glial cell lines. Peter Bogner, a postdoctoral fellow in my laboratory, is investigating the role of ATP and other nucleotides in cell adhesion.

Publications

Friedman SJ, Skehan P. Neoplasms, etiology. In: Dulbecco R, ed. Encyclopedia of Human Biology, vol 5. Academic Press, 1990.

Skehan P, Friedman SJ. Tissue sizing a community control of neoplastic growth. In: Paukovits RW, ed. Growth regulation and carcinogenesis. CRC Press, 1990.

Skehan P, Friedman SJ. Cancer - a disease of tissue neogenesis. In: Paukovits RW, ed. Growth regulation and carcinogenesis. CRC Press, 1990.

Skehan P, Kenney S, Bokesch H, Friedman SJ. A 3-dimensional anticancer colony extinction assay for plastic attached cells, Cell Tissue Kinet 1990 in press.

Bai R, Pettit G, Friedman S, Hamel E. The cytotoxic peptides of Dolabella auricularia: cellular and biochemical studies, Proc Am Assn Cancer Res 1990 in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06199-01 LBC

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pyrimidine Nucleotides and Extracellular Matrix Synthesis

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

PI:	R. Cysyk	Pharmacologist	LBC NCI
Others:	C. Chisena	Biologist	LBC NCI
	K. Duncan	IRTA	LBC NCI
	M. Grubb	IRTA	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:
3.5	2.0	1.5

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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.)*

Activation of uridine uptake is an early event in the mitogenic response, occurring within minutes of mitogenic stimulation of quiescent 3T3 mouse fibroblasts, compared with DNA synthesis which occurs 10-15 hours later. Data from our Laboratory indicate that the early activation of pyrimidine nucleotide synthesis is linked to the synthesis of components of the extracellular matrix (specifically, hyaluronate). Hyaluronate has been implicated as a factor involved in tumor invasion and metastasis. Thus elucidation of cellular regulatory mechanisms that control the synthesis of oligosaccharide moieties of extracellular matrix components could be useful to the discovery of agents with anti-invasive and anti-metastatic properties.

Chromatographic analysis of the nucleotide pool in 3T3 fibroblasts 30 minutes after serum stimulation revealed an increase in UDP-glucuronide that corresponded to the increase in uridine uptake with regard to time course and magnitude. Other factors capable of stimulating uridine uptake, including EGF, PDGF, IL-1, and phorbol ester also caused an increase in UDP-glucuronide. Certain growth factors and various combinations of growth factors stimulated 3T3 fibroblasts to secrete hyaluronate. Of particular interest was the finding that conditioned media from a BT-20 human breast carcinoma (whose oncogene product is PDGF) stimulates hyaluronate synthesis by fibroblasts indicating intercellular communication between tumor and normal fibroblasts in the synthesis of hyaluronate induced by this human tumor.

Studies into the biochemical mechanism for mitogen-stimulation of hyaluronate synthesis indicate that UDP-glucose dehydrogenase activity is increased following mitogen-stimulation of quiescent fibroblasts. This stimulation is an early event following mitogenic activation. Current studies are focusing on the regulatory role of UDP-xylose in the synthesis of proteoglycans and glycosaminoglycans.

Objectives:

Quiescent fibroblasts, stimulated to proliferate by the addition of serum or defined mitogens, is a useful model system to study the sequence of events that intervene between mitogen-receptor interaction and initiation of DNA synthesis. Activation of uridine uptake is an early event in the mitogenic response, occurring within minutes of mitogenic stimulation compared with DNA synthesis which occurs 10-15 hours later. Data from our Laboratory indicate that the early activation of pyrimidine nucleotide synthesis is linked to the synthesis of components of the extracellular matrix. Thus, early activation of pyrimidine nucleotide synthesis may be related to a physiologic function unique to fibroblasts rather than a replicative event common to all cell types. Involvement of pyrimidine nucleotide synthesis in extracellular matrix formation may have particular relevancy to the control of tumor cell invasion and metastasis. This Project has the following Specific Aims:

1) Characterize the early activation of pyrimidine synthesis de novo in mitogen-stimulated 3T3 mouse fibroblasts and compare it with activation of purine base phosphoribosylation and activation of uridine uptake. Is the activation of pyrimidine synthesis de novo coordinately regulated with activation of purine nucleotide synthesis or uridine uptake?

2) Determine the relationship (if any) between early activation of pyrimidine nucleotide synthesis and hyaluronate formation. Is the early activation of pyrimidine nucleotide synthesis linked to a specific physiologic function of fibroblasts (i.e. extracellular matrix formation), or to a replicative event common to proliferating cells?

3) Design, identify, and evaluate agents to alter hyaluronate synthesis. Do these agents alter the early activation of pyrimidine nucleotide synthesis? Do agents that inhibit hyaluronate formation alter the invasive and metastatic properties of cancer cells in vivo?

Major Findings:

Correlation of mitogen-stimulated uridine uptake and UDP-glucuronide formation: In serum depleted medium, cultured fibroblasts will stop progressing through the cell cycle and become arrested in a G_1/G_0 state. When these quiescent cells are exposed to fresh serum or to a wide variety of other factors they resume cell cycle progression, leading to DNA synthesis 12-16 hours after the stimulation. This system has been widely used as a model to study control of cellular proliferation, with much attention focused on the biochemical events that occur within minutes of the stimulation. These early events are thought to be important links between the mitogenic signal and the cascade of biochemical changes leading to DNA synthesis and cell division. One of these early events is an increase in the uptake of uridine. This increased uptake is not due to an increase of uridine transport across the cell membrane, but is caused by increased phosphorylation of the cellular uridine. The connection between the mitogenic signal and the increased phosphorylation is not known. The apparent affinity for ATP of the phosphorylating system increases after serum stimulation, but no differences could be found in uridine kinase isolated

from stimulated or quiescent cells. Chromatographic analysis of the acid soluble nucleotide pool in 3T3 fibroblasts 30 minutes after serum stimulation revealed one component that was increased compared to quiescent controls. This component was identified as UDP-glucuronide by chromatographic and chemical means. The time course and magnitude of the serum stimulated increase in UDP-glucuronide is similar to the time course and magnitude of the increase in [^{14}C]uridine uptake. Other factors capable of stimulating [^{14}C]uridine uptake, including epidermal growth factor, platelet derived growth factor, interleukin-1, and a phorbol ester also caused an increase in UDP-glucuronide. The results show that one of the earliest changes in pyrimidine nucleotide metabolism after mitogen stimulation is an increase in UDP-glucuronide synthesis, which may be related to increased uridine uptake.

Growth factor stimulation of fibroblast hyaluronic acid synthesis: Many tumor cell lines secrete hyaluronic acid (HA), a polysaccharide that may facilitate the progression of solid tumors and metastases. Tumor cells may also stimulate HA secretion by normal cells through release of chemical mediators. Quiescent cultures of Swiss/3T3 fibroblasts were treated with serum-free media containing IL-1, PDGFaa, PDGFbb, EGF, insulin (INS), or 10% calf serum (CS). HA release was measured after 2-6 hours using ^{125}I-HA binding protein. HA production at 6 hours was INS=94$\pm$3, EGF=94$\pm$6, IL-1=152$\pm$9, PDGFaa=232$\pm$18, PDGFbb=226$\pm$4, CS=331$\pm$17 (% of HA release by serum free control $\pm$SEM). Conditioned media from BT-20 human breast carcinoma cells caused a 41$\pm$9% increase. PDGF and other factors released by human carcinomas may stimulate HA secretion by fibroblasts.

Mitogenic stimulation of UDP-glucose dehydrogenase activity in quiescent Swiss/3T3 fibroblasts: UDP-glucose dehydrogenase (UDPGDH: EC 1.1.1.22) plays an important role in glycosaminoglycan biosynthesis through regulating the conversion of UDP-glucose (UDPG) to UDP-glucuronic acid (UDPGA). A new HPLC method was developed to directly quantify the UDPGA formed by UDPGDH in cell homogenates. Using this method we have determined that enzyme activity increases significantly in homogenates from serum (10%) stimulated quiescent Swiss/3T3 fibroblasts. This rise occurred within 30 min. and continued for up to 8 hrs. until a plateau was reached. Kinetic parameters of UDPGDH in cultured log phase fibroblasts were determined at pH 8.7 and 37°C. The Km value for UDPG under saturating NAD concentrations was 166μM. UDP-xylose, a proposed intracellular regulator of UDPGDH, was shown to inhibit formation of UDPGA. The data indicate a role for UDPGDH in the early events following serum stimulation.

UDP-xylose synthesis in human lung cancer (A549) cells: Glycosaminoglycans are linked to proteoglycans via a galactosyl-galactosyl-xylosyl-serine unit. UDP-xylose serves to join xylose to the protein core serine residue. Xylose could be derived from extracellular sources, as it is able to enter the cell by the glucose transport system. Alternatively, UDP-xylose may be formed intracellularly through decarboxylation of UDP-glucuronic acid. UDP-glucuronic acid decarboxylase is present in some plant and bacterial cells, as well as in mouse mast cells. Additionally, UDP-xylose may play a regulatory role in glycosaminoglycan synthesis as it inhibits UDP-glucose dehydrogenase. UDP-sugars were extracted from human lung cancer cells (A549), the epimers separated via HPLC and UDP-xylose enzymatically degraded to xylose. To

determine the source of intracellular UDP-xylose, EI GC-MS of xylose derivatives was used. As TMS-forming reagents tend to react with both anomers, there were two peaks present in the chromatograms of these cyclic derivatives.

A method which traps the open-chain form of the sugar allowed for simpler quantitation. By reacting xylose first with hydroxylamine HCl and then with acetic anhydride, an open-chain derivative was formed. Electron ionization of this aldononitrile, peracetyl derivative yielded two fragments which comprise complementary pieces of the xylose carbon skeleton. By feeding cells uniformly C-13 labelled glucose, the pathway linking UDP-glucuronic acid to UDP-xylose can be determined.

Publications:

Zaharevitz DW, Chisena CA, Cysyk RL. Rapid increase of cellular UDP-glucuronide after mitogen stimulation of quiescent 3T3 mouse fibroblasts. Biochem Int 1990 in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07156-07 LBC

PERIOD COVERED
October 1, 1989 to September 30, 1990

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
	He Ruyi	Visiting 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:
2.5	2.5	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) study the metabolism of retinoic acid (RA), a potent inducer of differentiation; and b) 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. 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 thioester 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 differentiation 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.

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-ß-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 a thio-ester bond. Thus, the amino acid cysteine probably is the site 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. Studies on retinoylation use primarily two-dimensional gel electrophoresis as an analytical tool.

Major Findings

1. **The Monocytic Differentiation of HL60 Induced by Rat Kidney NADPH-Linked High-Km Aldehyde Reductase Protein.** The human promyelocytic leukemia cell line HL60 differentiates to monocyte/macrophage cells when incubated with NADPH-linked high-Km aldehyde reductase (EC 1.1.1.2) purified from the cytosol of rat kidney. Differentiation was assessed by cell growth, morphology, adhesiveness, nitroblue tetrazolium reduction, and nonspecific esterase activity. The extent of differentiation induced by the reductase and measured at four days by NBT reduction is dose-dependent with an ED50 (dose required for half-maximal effect) of 71 nM. In the presence of 10 nM retinoic acid the ED50 for reductase is reduced to 18 nM and an isobologram analysis of this effect indicates that the combination is synergistic. Inactivation of the enzymatic activity is not associated with a decrease in differentiation-inducing activity. These results suggest that the structure of the enzyme protein and not its enzymatic activity is involved with induction of differentiation. This view is supported by the demonstration that aldehyde reductase binds specifically to HL60 cells with a KD of 70 nM and that there are 13,000 binding sites/cell. Thus, the extent of differentiation induced by various concentrations of aldehyde reductase are directly related to the expected level of receptor occupancy.

2. **Covalent Binding of 17ß-Estradiol and Retinoic Acid to Proteins in the Human Breast Cancer Cell Line MCF-7.** Both retinoic acid and 17ß-estradiol formed covalent bonds with proteins of the human breast cancer cell line MCF-7. Two-dimensional gel patterns of the labeled proteins were unique for each ligand. There were four major retinoylated proteins in MCF-7 consisting of two doublets with molecular masses of 37 kDa and 20 kDa. These protein were designated 37a, 37b, 20c, and 20d. The extent of retinoylation was very low in a 55 kDa protein that we previously identified in the human myeloid leukemia cell line HL60 [Takahashi N, Breitman TR. J Biol Chem 1989;264:5159-5163]. These results indicated that the protein substrates for retinoylation may vary from cell-type to cell-type. About 10 proteins were labeled from 17ß-estradiol. Two of these proteins had mobilities that were identical to the retinoylated proteins 37a and 20c. These results indicate that in MCF-7 cells there are two proteins that can be retinoylated and labeled from estradiol. The demonstration that some ligands of the steroid/thyroid receptor family are covalently linked to cellular proteins suggests new mechanisms for the many effects of these agents on cells.

3. **Retinoylation of HL60 Proteins: Comparison to Labeling from Palmitic and Myristic Acids.** Recent studies suggest that a retinoic acid (RA) nuclear receptor or a retinoylated nuclear protein may be involved in the action of RA. We showed previously (Takahashi N, Breitman TR. J Biol Chem 1989, 264: 5159-5163) that retinoylation involves the formation of a thioester bond and occurs on protein in newly formed cells and in pre-existing cells. In the present study we saw at least 14 retinoylated proteins in HL60 cells. Greater than 90% of the retinoylation was associated with the nuclear protein described previously. This protein, partially purified from isolated nuclei, bound to DNA-cellulose and was eluted with NaCl. Retinoylation occurred in HL60 cells exposed to cycloheximide. Thus, retinoylation resembled palmitoylation, both in the covalent bond and the exchangeable reaction involving preformed protein. These similarities prompted us to compare retinoylation with two other fatty

acylations in growing HL60 cells. We found that the major retinoylated protein was labeled from either radioactive palmitic acid ([^{3}H]PA) or myristic acid ([^{3}H]MA). The extent of [^{3}H]PA labeling of this protein was not reduced by growth in the presence of RA. The extent of retinoylation of this protein was not reduced by growth in the presence of increasing concentrations of PA. These results raised the possibility that the same protein was a substrate for retinoylation, palmitoylation, and myristoylation.

4. **Retinoylation: Occurrence in Cell Lines with Varied Responses to Retinoic Acid.** In HL60 cells a nuclear protein with a Mr of about 55,000 is retinoylated, probably with the formation of a thioester bond (Takahashi, N., and Breitman, T. R. (1989) J. Biol. Chem. 264, 5159-5163). To gain further knowledge on the role of retinoylation we studied it in cell lines with varied responses to retinoic acid (RA). Compared to HL60 the extent of retinoylation (mol/cell) was about five-fold higher in HL60/MRI, a mutant which is more sensitive to RA than HL60. Retinoylation occurred to the same extent in HL60 and in a mutant, HL60/RA-res, which is resistant to differentiation by RA. The two-dimensional polyacrylamide gel electrophoresis patterns of these three cell lines were different. While we saw the same major retinoylated protein of Mr 55,000 in the three cell lines, the HL60/RA-res cells also contained a high level of a protein with the same Mr and a lower pI. The extent of retinoylation was greater in a RA-sensitive embryonal carcinoma cell line (PCC4.azalR) than in a RA-resistant cell line (PCC4.(RA)$^-$2). One-dimensional polyacrylamide gel electrophoresis patterns of retinoylated proteins of both cell lines were different from HL60. Also, PCC4.azalR cells had retinoylated proteins that we did not see in PCC4.(RA)$^-$2 cells. The retinoylation pattern of the normal canine kidney cell line (MDCK) was different from either HL60 or the embryonal carcinoma cells. These results showed that retinoylation was widespread and that the response to RA of different cell types may depend on the retinoylation of specific proteins.

5. **Combinations of Retinoic Acid with either Sodium Butyrate, Dimethylsulfoxide, or Hexamethylene Bisacetamide Synergistically Induce Differentiation of the Human Myeloid Leukemia Cell Line HL60.** All-trans-Retinoic acid (RA), sodium n-butyrate (NaB), hexamethylene bisacetamide (HMBA) and dimethyl sulfoxide (DMSO) induce differentiation of the human acute myeloid leukemia cell line HL60. In the clinic, RA, NaB, or HMBA induce complete or partial remissions. However, the achievement and maintenance of effective plasma concentrations and toxicity have been problems. These difficulties led us to study the interaction of RA with these inducers. We found that combinations of RA with either NaB, HMBA, or DMSO synergistically induced terminal differentiation of HL60. A measure of the effectiveness of these combinations was that the doses of NaB, HMBA, and DMSO required alone to induce half-maximal differentiation were decreased about fourfold in combination with normal plasma concentrations of about 30 nM RA. RA or NaB alone did not enhance the growth of HL60 cells. In contrast, HMBA or DMSO alone increased growth of HL60 cells even at concentrations that did not induce differentiation. The addition of RA reduced the promotion of growth and increased the extent of terminal differentiation seen with HMBA and DMSO alone. These data suggest that treatment of some malignancies with combinations of RA with HMBA or NaB may maintain differentiation-inducing effects and decrease the problems associated with the

achievement and maintenance of effective plasma concentrations as single agents.

6. **Retinoic Acid Inhibits Sodium Butyrate-Induced Monocytic Differentiation of HL60 Cells While Synergistically Inducing Granulocytoid Differentiation.** The human myeloid leukemia cell line HL60 is widely used as an in vitro model to study myeloid differentiation. HL60 cells differentiate along different cell type lineages in response to a variety of compounds. The direction of differentiation is generally inducer specific. Thus, all-trans-retinoic acid (RA) and dimethylsulfoxide induce granulocytic differentiation and phorbol esters and 1,25-dihydroxyvitamin D3 induce monocyte/macrophage differentiation. However, the response of HL60 cells to sodium n-butyrate (NaB) is pleiotropic. NaB induces HL60 along the monocytic, neutrophilic, eosinophilic, and basophilic pathways. In this study we saw that physiologic concentrations of RA switched the direction of NaB-induced differentiation from monocytic to granulocytic. Thus, after 4 days of exposure to 600μM NaB, about 80% of HL60 cells were nonspecific esterase (NSE)-positive with a monocyte morphology. After exposure of HL60 cells to a combination of 600μM NaB and 30 nM RA, we saw 26% NSE-positive cells, 12% monocytes, and 69% mature (banded or segmented) neutrophils. With 30 nM RA alone we saw 10% mature neutrophils and no NSE-positive cells or monocytes. With 1μM RA we saw 35% mature neutrophils. A previous study (TR Breitman and R-Y He, Cancer Res in press) showed that combinations of RA and NaB synergistically induce HL60 to cells that reduce nitroblue tetrazolium. The present study demonstrated that this synergy was even greater if the parameter measured was mature granulocytes. Our results indicated that the endogenous RA in the serum used to grow cells in culture may markedly affect the direction of differentiation of HL60 cells induced by NaB. Furthermore, our results may provide additional rationale for the use of combinations of RA and NaB in the treatment of some malignancies.

Proposed Course

1. In the retinoylation subproject we will continue to examine other cell lines for the presence of retinoylated proteins. Of more immediate interest is the identification of the retinoylated proteins. It is likely that many of these proteins are known. The fact that they are retinoylated should allow us to better put together the function that retinoylation plays 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 extend these studies by examining the interrelationship of RA with other inducers (e.g., vitamin D3) and to see if fresh cells from leukemia patients respond in vitro to these combinations.

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ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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.

CPE-C, the cytidine analogue of the natural product, neplanocin, was synthesized by the LMC several years ago. This compound should enter clinical trial as an anticancer agent late this year. A significant amount of preclinical analytical and biological work has been accomplished as support for the Phase I clinical trial. In addition, the LMC continues to explore the fruitful area of carbocyclic synthesis.

3-Deaza-CPE-U, although less cytotoxic than CPE-C, is, surprisingly, more cytotoxic than 3-deaza-CPE-C. 5-Aza-CPE-C is significantly more hydrolytically stable than 5-azacytidine and is being evaluated biologically. An LMC total synthesis produced the natural product, neplanocin F. This compound was without L1210 cytotoxicity. Psicoplanocin, a hybrid of neplanocin and psicofuranine (a naturally occurring GTP inhibitor), was synthesized and is under evaluation for its anticancer properties.

3-Deazaneplanocin A, a compound previously synthesized in this laboratory and the best known inhibitor of S-adenosylhomocysteine hydrolase, was found to possess potent activity against the monkey ebola virus.

2′-Fluoro-dideoxynucleosides (F-ddN) are of practical interest as anti-HIV agents because of their acid-stability and potential as orally administered drugs. It is possible that two LMC compounds in this series will enter clinical trial after license to pharmaceutical companies. Compounds in this LMC-discovered series continue to be pursued. A new method of synthesis of these compounds was completed which greatly simplifies the preparation of F-ddN analogues.

In an attempt to prepare compounds with improved central nervous system (CNS) penetration, a F-ddN series was designed which had increased lipophilic character. Four of ten 6-substituted F-ddN were protective against HIV in the ATH8 system but, thus far, the compounds prepared have not been as active as their non-fluorinated parent compounds.

Oxetanocin, a naturally occurring nucleoside with a four-member sugar, has anti-HIV activity. The LMC expanded this unusual sugar to a 5-member ring with retention of activity. Currently, structure-activity studies based on the sugar, apiose, is underway. This series is of particular interest since there are theoretical reasons why the alpha anomers may be active.

An NMR mechanistic study has identified a decomposition product which may be responsible for the preclinical antitumor and antiviral activity of pyrimidin-2-one riboside (zebularine).

Protein kinase C (PK-C) inhibitors are being pursued through the synthesis of chiral pentonolactones for structure-activity studies. We have established the most likely conformation of diacylglycerol when this naturally occurring agonist binds to the regulatory site of PK-C. A number of fatty acid esters were synthesized and evaluated for their ability to displace radiolabeled phorbol ester from its binding site. We expect the newest analogues synthesized to have even lower K_i values than the best achieved, thus far (2.7 μM).

As part of our project to design and synthesize inhibitors of tyrosine kinase (TK), a number of phosphonate-containing compounds were prepared along with some literature compounds claimed to be inhibitory. The particular TK test system employed appears to be very important relative to compound activity. An important finding is that one compound synthesized inhibits TK (lck) autophosphorylation without affecting exogenous substrate phosphorylation.

A specially synthesized reagent was used to replace cytidine residues with dihydro-5-azacytidine in specific locations in synthetic 26-mer oligonucleotides.

Computerized molecular modeling techniques are used routinely to assist in the selection of new anticancer and anti-AIDS molecules for synthesis. Our two Silicon Graphics workstations are in full-time use in collaboration with synthesis chemists on the following projects - (1) design of competitive inhibitors of protein kinase C, (2) design of dideoxynucleosides based on the active members of the series and (3) quantitative structure-activity studies on inhibitors of tyrosine kinase. Additionally, the conversion of the DTP chemistry data base (DIS) from two- to three-dimensional structures is being explored.

A large amount of analytical chemistry research has been devoted to supporting the projected anticancer clinical trial of cyclopentenyl cytosine (CPE-C), a LMC carbocyclic nucleoside. CPE-C pharmacokinetics were determined following continuous infusions in dogs and mice. Parent drug was still detected 24 hours after infusion termination indicating that substantial drug is sequestered in cells, probably as the relatively stable triphosphate.

In a rhesus monkey study, deamination of CPE-C to CPE-U occurred. This was not observed in dogs, rats or mice. Using fresh kidney and liver samples, tissue concentrations of cytidine deaminase were as found follows - monkey > human > mice = dogs > rats.

In MOLT-4 cells, nine CPE-C metabolites were detected. CPE-C triphosphate was a potent inhibitor of CTP synthetase (75 nM) in this system.

The human bioequivalence of solutions and tablets of hexamethylene bisacetamide (HMBA) was established in a Walter Reed collaborative Phase I/II trial.

In collaboration with COP, dideoxyguanosine (ddG) was studied as an anti-hepatitis drug in the duck model. Pharmacokinetics and bioavailability (up to 85%) were determined for different administration sites.

2′-F-dd-ara-C and ddC were equally stable to cytidine deaminase in monkey plasma (14% decomposition in 28 hours).

Six 2′-F-dd-purines were characterized as substrates for adenosine deaminase (ADA) by V_{max} and K_m values in vitro. These 2′-fluoro anti-AIDS agents deaminate much more slowly than adenosine or ddA. 6-Halo-dd-purine nucleosides were found to require ADA hydrolysis (to inosine analogues) in order to produce their anti-HIV effects.

A rapid, microscale (1 mg) octanol-water partition coefficient method was developed. Over 70 nucleosides have been evaluated. This type of data is particularly useful for the design of drugs for treatment of disease in the central nervous system.

Mass spectral research has characterized the matrix and halogen characteristics which facilitate the unexpected reductive dehalogenations observed during FAB/MS analysis of halonucleosides.

The HIV virally-encoded gag-pol polyprotein contains a 99-residue segment that acts as a virus-specific protease. This enzyme activity is necessary for the maturation of the HIV virus. Using the "sense/antisense" concept, 42 polypeptides were designed and synthesized in an attempt to inhibit five different protease regions. Most of the polypeptides contained 12-15 amino acid residues. The best inhibitor of the isolated enzyme discovered this far (Ki = 155 μM) is directed towards the "flap region". Thirteen of the 42 compounds tested have produced some inhibition of this key HIV enzyme.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 03581-21 LMC

PERIOD COVERED
October 1, 1989 to September 30, 1990

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
	Pamela L. Russ	Chemist	LMC, NCI
	Harry Ford, Jr.	Biotechnology Fellow	LMC, NCI
	Lajos Hegedus	Visiting Fellow	LMC, NCI
	Fuming Xie	Visiting Fellow	LMC, NCI

COOPERATING UNITS *(if any)*
Laboratory of Biochemical Pharmacology, DTP, DCT; Toxicology Branch, DTP, DCT; Pediatric 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:
2.2	1.5	0.7

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.)*

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 cyclopentenyl cytosine (biochemistry, preclinical pharmacology and methods development), carbodine/isocarbodine (synthesis and analytical characterization), hexamethylene bisacetamide (tablet bioequivalence and pharmacokinetics) and modified nucleosides (analytical characterization).

GPO 914-918

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.

Major Findings:

1. Bioequivalence, Metabolism and Pharmacokinetics of Oral Hexamethylene Bisacetamide (HMBA) as a Tablet Dosage Form in an Adult Phase I Clinical Trial(Drs. Kelley, Chun, Ward, Ms. Roth): Hexamethylene bisacetamide (HMBA, NSC 95580), a potent in vitro differentiating agent, has been evaluated in a collaborative (NCI and Walter Reed Army Medical Center) Phase I clinical trial to determine the bioequivalence of 1 gm tablets to a 5% drug solution during a 5-day period of repetitive oral administration. Tablet HMBA was bioequivalent to solution HMBA in terms of plasma levels, total AUC, metabolic pattern and urinary excretion.

2. Preclinical Pharmacology and Enhancement of Current Analytical Methods for Cyclopentenyl Cytosine (CPE-C) and its Metabolites:

a. Disposition and Pharmacokinetics of CPE-C in Small Animals (Drs. Kelley, Hegedus, Hartman, Tomaszewski, Zaharko, Ms. Roth): CPE-C (1, NSC 375575) pharmacokinetics are being examined following continuous infusion of the drug in athymic mice and in male beagle dogs in order to test the general pharmacokinetic model previously developed during bolus dose studies. For beagle dogs, steady-state plasma concentrations were achieved more slowly than predicted following 24- and 72-hr continuous infusions of CPE-C. CPE-C was still detectable, however, for 48 hr after the end of the 24-hr infusion to indicate that substantial drug is sequestered in the deep compartment even after low doses.

b. Disposition, Metabolism and Pharmacokinetics of CPE-C in Non-human Primates (Drs. Kelley, Hegedus, Blaney, Balis, Heideman, Poplack): A study to investigate CPE-C disposition, metabolism and pharmacokinetics in male rhesus monkeys has been completed. Following an iv bolus dose of 100 mg/m^2, plasma elimination of CPE-C was biexponential with a mean terminal phase half-life of only 36 min. The mean plasma clearance of for monkeys was 5- to 10-fold more rapid than clearance rates in rodents and dogs. This more rapid clearance is due primarily to deamination of CPE-C in the primate and results in a much lower total drug exposure as measured by AUC. Cyclopentenyl uracil (CPE-U, 2) was the major

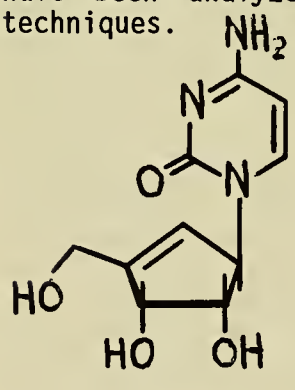
plasma metabolite and urinary excretion product, with less than 20% of the administered dose of CPE-C being recovered as unchanged drug. CPE-U plasma levels exceeded those of CPE-C within 20 min. CPE-U also exhibited approximately 4X better central nervous system penetrability. A 24-hr continuous infusion of CPE-C at a rate of 12.5 mg/m^2/hr was well tolerated and achieved average steady-state CPE-C and CPE-U plasma concentrations of 2.1 μM and 8.2 μM, respectively.

c. Enhancement of Analytical Methods for Measuring CPE-C in Biological Fluids (Drs. Kelley, Xie): Biological sample preparation procedures were further refined to allow nearly quantitative isolation of CPE-U. Research was also initiated to enhance the detectability of CPE-C, since anticipated Phase I clinical trials employing short continuous infusions will probably result in plasma steady-state levels of drug below the current limit of quantitation of 0.1 μM. Fluorescence detection following derivatization to produce a substituted cyclopentenyl ethenocytosine (3) is currently under investigation.

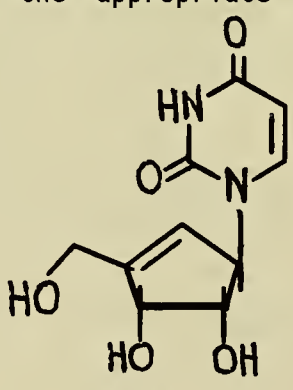
d. Biochemical Studies with CPE-C (Drs. Ford, Kelley, Cooney, Ahluwalia, Johns, Ms. Roth): Relative cytidine deaminase levels were measured in mammalian plasma to assess the potential of various species to metabolize CPE-C to CPE-U. Highest enzyme levels were found in rhesus monkeys, followed by humans, BDF_1 mice, and dogs with Sprague-Dawley rats being the lowest. The cellular pharmacology and metabolism of CPE-C was also investigated in Molt-4 lymphoblasts, a human T-cell line. Nine CPE-C metabolites were detected. CPE-CTP was an extremely potent inhibitor of CTP synthetase in this system with an IC_{50} of only 75 nM. Since deamination of CPE-C by Molt-4 cytidine deaminase could not be demonstrated, deamination of CPE-CTP by cytidylate deaminase is currently being investigated.

3. Automation of Laboratory Chromatography Capabilities Through Networked Personal Computers (Drs. Kelley, Musser, Ms. Roth): Capabilities for graphics, pharmacokinetic analysis and word processing have been added to a personal computer (PC) based, multi-tasking chromatography data system. Efforts are also currently underway to connect the mass spectrometer data system, based on a PDP 11/73 minicomputer, to laboratory PC's for mass spectral file transfer.

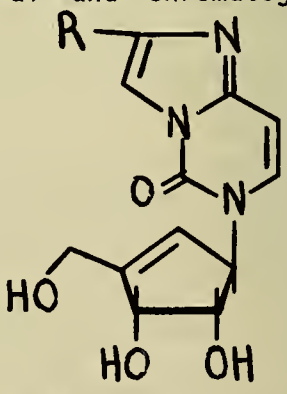
4. Synthetic and Collaborative Project Support (Drs. Kelley, Ford, Ms. Russ): Numerous samples which cannot be categorized as coming from any one project area have been analyzed by the appropriate mass spectral and chromatographic techniques.



1 (CPE-C)



2 (CPE-U)



3 (etheno-CPE-C)
R = H, CH_3, phenyl, napthyl

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 1989; 12: 1613-1633.

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 1989; 49: 5213-5216.

3. Surbone A, Ford H Jr, Kelley JA, Ben-Baruch N, Thomas RV, Fine R, Cowan, KH. A Phase I and pharmacokinetic study of arabinosyl-5-azacytosine (fazarabine, NSC 281272). Cancer Res 1990; 50: 1220-1225.

4. Zaharko DS, Kelley JA, Tomaszewski JE, Hegedus L, Hartman NR. Cyclopentenyl cytosine: Interspecies predictions based on rodent plasma and renal kinetics. Inv New Drugs, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06173-05 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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	Deputy Chief	LMC, NCI
Others:	J. S. Driscoll	Chief	LMC, NCI
	J. Barchi	Senior Staff Fellow	LMC, NCi
	R. Wysocki	IRTA Fellow	LMC, NCI
	M. Siddiqui	Technician	LMC, NCI
	P. Russ	Technician	LMC, NCI

COOPERATING UNITS *(if any)*

Toxicology Branch, DTP, DCT, NCI
DTP Anti-HIV Testing Program, FCRF
OAD, 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:
2.75	2.50	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 methodology for the synthesis of hydrolytically stable dideoxy nucleosides, considered to be of potential use as anti-HIV agents, has been extended to various modified purine and pyrimidine bases. The 2'-fluoro substituent in the ara-configuration protects these compounds against chemical as well as enzymatic degradation. Some of the new compounds synthesized appear to function as "prodrugs" which require the action of adenosine deaminase to uncover their biological activity.

A new synthetic approach toward fluorine-containing dideoxynucleosides was developed. It greatly facilitates the process since a critical reductive step is now performed before the condensation reaction. This reaction is used only once for the production of many compounds, rather than every time a new compound is prepared.

A series of dideoxyapiose nucleosides is being developed as potential anti-HIV agents. These compounds were designed as partial structures of the ring expanded analogue of the antibiotic oxetanocin.

Project Description:

General Objective:

The objective of this project is the discovery of effective and stable 2′,3′-dideoxynucleoside analogues with potent anti-HIV activity.

Specific Objectives:

1. Synthesis of acid-stable purine and pyrimidine dideoxynucleoside analogues.

2. Synthesis of sugar-modified dideoxynucleoside analogues.

3. Synthesis of CNS-active "pro-drug" purine dideoxynucleoside analogues with increased lipophilicity.

Major Findings:

Synthesis of Acid-Stable Dideoxypurine and Pyrimidine Nucleosides (Dr. Marquez, Dr. Barchi, Mr. Siddiqui, Dr. Driscoll):
The discovered hydrolytic and enzymatic stability of the anti-HIV active agents, 9-(2,3-dideoxy-2-fluoro-ß-D-threo-pentofuranosyl)adenine and hypoxanthine [2′-fluoro-ara-ddA (1) and 2′-fluoro-ara-ddI (2), respectively], which is directly related to the presence of the fluorine atom in the sugar moiety, was the subject of further studies. Other important fluorine-containing dideoxy nucleosides with bases such as 6-chloropurine (3), guanine (4), 2-amino-6-chloropurine (5), and cytosine (6), were synthesized and biologically evaluated.

The cytosine analogue (6) was very effective and considered "active" by the DTP anti-HIV testing program of the NCI (EC_{50} 1.01×10^{-6} M in CEM-6 cells infected with HIV). This compound was equally considered "active" in the ATH-8 system (COP).

The rest of the purine analogues (3-5) displayed levels of activity that were inferior to those of 1, 2 and 6. The role of adenosine deaminase (ADA) as a key enzyme in generating the active species 2 and 4, respectively, from 3 and 5 was studied. Experiments in the presence of an ADA inhibitor (2′-deoxycoformycin) suggested that there might be an important role for ADA in the activation of these agents; however, addition of ADA did not produce the expected increased biological activity.

A new and more direct synthetic approach to the synthesis of fluorine-containing dideoxynucleosides was developed. The key intermediate 7 was synthesized and coupled with 6-chloropurine to give exclusively the desired β-anomer, 3. Compound 3 was converted in one step to 2′-fluoro-ara-ddA (1).

A rapid method for the synthesis of the triphosphate metabolite of 2′-F-ara-ddA was developed.

Synthesis of Sugar-Modified Dideoxynucleoside Analogues (Dr. Wysocki, Dr. Marquez): Following the previously discovered lead that a ring-enlarged analogue, 8, of the anti-HIV active natural product, oxetanocin (9), possessed essentially the same level of activity in ATH-8 cells, the synthesis of a number of partial structures derived from 8 was undertaken. Since removal of one of the hydroxymethyl groups from 8 produces the very potent dideoxyadenosine (ddA), the importance of the other hydroxymethyl group is expected to be deduced from the activity of the ddA isomer 10. This compound was synthesized from commercially available protected apiose in 14 steps and it represents the first dideoxyapiose nucleoside ever made. Anti-HIV evaluation is in progress. Interestingly, the α-anomer of this compound (11), is isosteric to ddA. The synthesis of 11 is in progress.

Synthesis of Potentially CNS-Active Purine Analogues with Increased Lipophilicity (Dr. Driscoll, Dr. Barchi, Dr. Marquez): In an attempt to explore the hypothesis that the dideoxynucleoside series has an optimum partition coefficient for CNS penetration, a series of purine analogues was designed and ten members synthesized and tested. While a number had anti-HIV activity and were more lipophilic than AZT, none possessed a level of activity high enough to enter the next step - determination of CSF/plasma ratios in monkeys. 2′-F-6-Cl-dd-ara-purine gave >60% protection from HIV-1 in ATH8 cells and had log P = 0.32.

Publications:

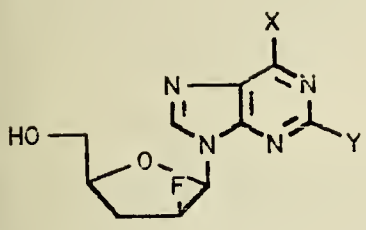
Marquez VE: The Design, Synthesis, and Antiviral Activity of Nucleoside and Nucleotide Analogues. ACS Symposium Series 401, Martin, J. C, ed., American Chemical Society, Washington DC, 1989, pp 140-155.

Marquez VE, Tseng CKH, Mitsuya H, Aoki S, Kelley JA, Ford Jr H, Roth JS, Broder S, Johns DG, Driscoll JS: Acid-Stable 2′-Fluoro Purine Dideoxynucleosides as Active Agents against HIV. J Med Chem 1990; 33: 978-985.

Masood R, Ahluwalia GS, Cooney DA, Fridland A, Marquez VE, Driscoll JS, Hao Z, Mitsuya H, Perno CF, Broder S, Johns DG: 2′-Fluoro-2′,3′-dideoxyarabinosyladenine: A Metabolically Stable Analogue of the Antiretroviral Agent 2′,3′-Dideoxyadenosine. Mol Pharmacol 1990; 37: 590-596.

Marquez VE; Effect of Fluorine Substitution on the Anti-HIV Activity of Dideoxynucleosides. Current Chemical and Pharmacological Advances on Drugs of Abuse which Alter Immune Function and their Impact upon HIV Infection. NIDA Research Monograph #96, 1990, pp 61-79.

Tseng CKH, Marquez VE, Milne GWA, Mitsuya H, Shirasaki T, Wysocki Jr RJ, Driscoll JS: A Ring-Enlarged Oxetanocin A Analogue as an Inhibitor of HIV Infectivity. J Med Chem, in press.



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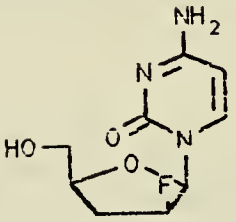

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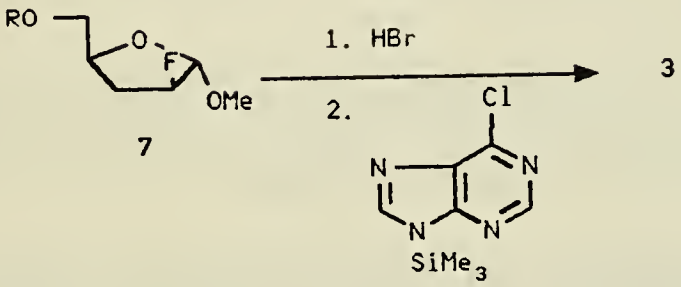

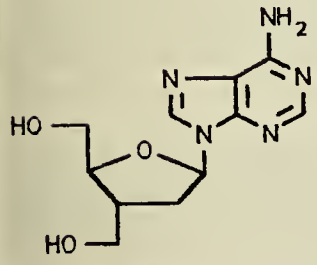

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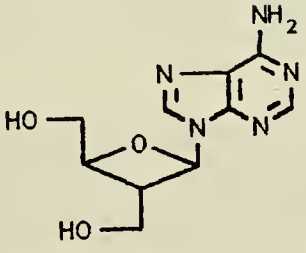

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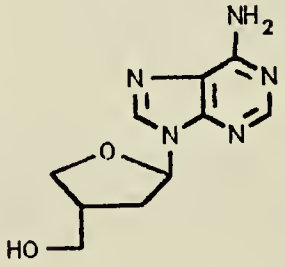

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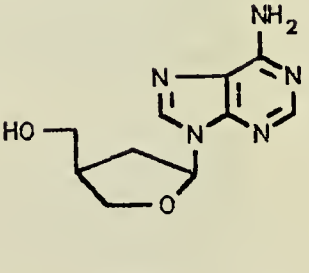

11

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06174-05 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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		Deputy Chief	LMC, NCI
Others:	John S. Driscoll	Chief	LMC, NCI
	Michael Bodenteich	Visiting Fellow	LMC, NCI
	Benjamin B. Lim	IRTA Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Biochemical Pharmacology, DTP, DCT, NCI
Southern Research Institute, Birmingham, Alabama.
USAMRIID, Fort Detrick, Maryland

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

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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.)*

Several novel analogues of the potent antitumor agent cyclopentenyl cytosine (CPE-C) were synthesized. The modifications consisted mainly in changes in the aglycon moiety with the intent to enhance antimetabolite activity. These changes included the preparation of the corresponding 3-deaza- and 5-azapyrimidine analogues which are known in the conventional riboside series to possess antimetabolic and antitumor activity.

The first synthesis of a carbocyclic ketosugar nucleoside analogue was accomplished. The prototypic compound of this series is psicoplanocin A, which incorporates in one molecule the structural features of the antibiotics neplanocin A and psicofuranine. The corresponding cytosine and uracil analogues were also prepared.

3-Deazaneplanocin A, a compound previously synthesized in this laboratory and the best known inhibitor of S-adenosylhomocysteine hydrolase, was found to possess potent activity against the ebola virus.

Project Description

Objective: The objective of this work is the systematic synthesis and evaluation of modified cyclopentenyl carbocyclic nucleosides as antitumor and antiviral agents. These compounds are structurally related to the naturally occurring antibiotic neplanocin A.

Major Findings:

CPE-pyrimidine analogues (Dr. Lim, Dr. Marquez): The target carbocyclic 3-deazacytosine and 3-deazauracil nucleosides (2 and 3) were evaluated in vitro for cytotoxicity (L1210 leukemia) and antiviral activity against DNA (HSV-1) and RNA (Human Influenza) viruses. The 3-deazacytosine analogue (2) was nontoxic to L1210 cells, while the 3-deazauracil analogue (3) displayed significant but reduced cytotoxicity in relation to CPE-C (1). Both compounds were also devoid of antiviral activity. A new and more efficient synthesis was devised for the corresponding carbocyclic 5-azacytosine analogue (4). This compound proved to be significantly more stable than its riboside counterpart and it is currently being evaluated for biological activity. The corresponding 5-azauracil nucleoside (5) was also synthesized but its isolation proved difficult due to the instability of the compound. However, the corresponding 4-methoxy analogue (6) was isolated as a stable compound.

Carbocyclic nucleoside analogues derived from a modified cyclopentenyl moiety (Dr. Bodenteich, Dr. Marquez): The target compound, neplanocin F (7), synthesized last year, was evaluated in vitro for cytotoxicity (L1210 leukemia) and antiviral activity against HSV-1 and human influenza. No useful activity was detected.

The synthesis of the first carbocyclic ketosugar nucleoside was accomplished. The compound, psicoplanocin A (8), combines in one molecule the structural features of two natural products, neplanocin A and psicofuranine. Psicofuranine (9) is an antibiotic that inhibits GMP synthetase but it is rather unstable to acidic or basic conditions. The syntheses of the corresponding carbocyclic analogues of 1-β-D-psicofuranosylcytosine (10) and uracil (11) was also accomplished. The biological evaluation of these compounds is in progress.

CPE-purine analogues (Dr. Marquez, Dr. Driscoll): 3-Deazaneplanocin A (3DN), a compound previously synthesized by the LMP, was evaluated as an inhibitor of ebola virus in vitro. This virus is responsible for the ban on importation of monkeys for research by several jurisdictions. The U.S. Army at Fort Detrick has found 3DN to be a potent inhibitor of ebola and has received our synthetic procedure in order to pursue a large scale synthesis. They plan to conduct in vivo studies with the compound.

Publications:

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 1989; 32: 1442-1446.

De Clercq E, Cools M, Balzarini J. Marquez VE, Borcherding DR, Borchardt RT, Drach JC, Kitaoka S, Konno T: Broad-Spectrum Antiviral Activities of Neplanocin A, 3-Deazaneplanocin A, and Their 5′-Nor Derivatives. Antimicrob Ag Chemother 1989; 33: 1291-1297.

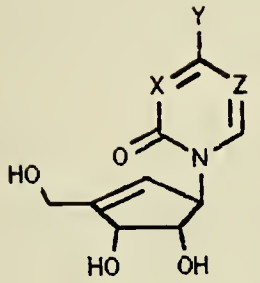
Driscoll JS, Marquez VE, Plowman J: Cyclopentenyl Cytosine (CPE-C). A Carbocyclic Nucleoside with Antitumor and Antiviral Properties. Nucleosides Nucleotides 1989; 8: 1131-1133.

Bodenteich M, Marquez VE: Total Synthesis of (+/-)-Neplanocin F. Tetrahedron Lett 1989; 30: 4909-4912.

Kim SK, Fuller RW, Marquez VE: Synthesis and Biological Activity of Ara and 2′-Deoxycyclopentenyl Cytosine Nucleoside Analogues. Nucleosides Nucleotides, in press.

Marquez VE, Lim, M-I, Khan MS, Kaskar B. (4R,5R)-(-)-3-Benzyloxymethyl-4,5-O-isopropylidene-2-cyclopentenone. An Optically Active α,ß-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.

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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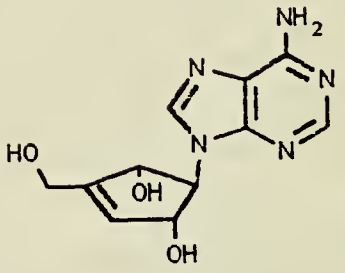

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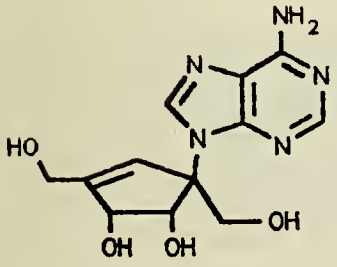

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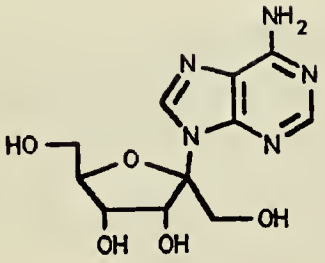

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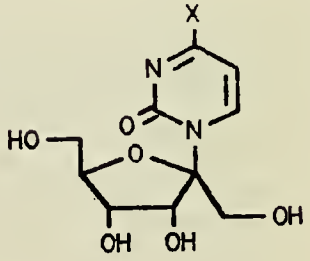

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06175-05 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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	Deputy Chief	LMC, NCI
Others:	Estela Alvarez	IRTA Fellow	LMC, NCI
	Harry Ford	Biotechnology 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	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 synthesis of oligodeoxynucleotides containing a dihydro-5-azacytosine moiety instead of cytosine at any desired position of a sequence in an oligo can now be achieved routinely. A new and efficient preparative HPLC method for the purification of these oligodeoxynucleotides was also developed. Oxidation of the dihydro-5-azacytosine moiety to the aromatic 5-azacytosine has been attempted and continues to be investigated. The target oligodeoxynucleotides containing 5-azacytosine moieties are expected to behave as inhibitors of DNA methylase and to affect gene expression.

Project Description

Objective: 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 discern the relationship between DNA methylation and gene expression.

Major Findings:

Synthesis of DHAC-modified 26-mers (Dr. Alvarez, Dr. Ford, Dr. Marquez): The synthesis of modified 26-mers was accomplished. The general structure of these oligomers is as follows: 5′-CCGGCCATTACGGATCCGTCCTGGGC-3′. The successful replacement of one of the underlined cytosines (C) in a single oligonucleotide led to three different 26-mers. A new protocol was developed for the successful coupling of the DHAC phosphoramidite during the DNA synthesis and also a new HPLC purification procedure was developed to give final products with excellent purity (judged by gel electrophoresis of the ^{32}P-labeled oligonucleotides).

Oxidation of DHAC to AC in the DHAC-modified 26-mers (Dr. Alvarez, Dr. Marquez): The specific sequence of the 26-mer (vide supra) was selected on the basis of the recognized substrate properties of this parent oligonucleotide for DNA methylase. If the conversion of the DHAC moiety to the aromatic AC moiety is successful, the resulting oligomers have the potential to behave as inhibitors of DNA methylase. This conversion was successful when applied to dimers and now the procedure has been applied to each of the DHAC-containing 26-mers. At this point preliminary results are encouraging; however, the extent and efficiency of the oxidation is still under investigation.

Publication:

Goddard AJ, Marquez VE: Synthesis of Oligonucleotides Containing 5,6-Dihydro-5-azacytosine and 5-Azacytosine at Specific CpG Sites. Nucleosides Nucleotides 1989, 8: 1015-1018.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06176-05 LMC

PERIOD COVERED
October 1, 1989 to September 30, 1990

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		Deputy Chief	LMC,NCI
Others:	John S. Driscoll	Chief	LMC,NCI
	Kelly Teng	IRTA	LMC,NCI
	Joseph J. Barchi	Sr. Staff Fellow	LMC,NCI

COOPERATING UNITS *(if any)*
Laboratory of Biochemical Pharmacology, DTP, DCT, NCI; University of North Carolina, Chapel Hill, NC; Laboratory of Carcinogenesis and Tumor Promotion, DCE, NCI.

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	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 chemical structures of the first and last products formed during the base-catalyzed decomposition of zebularine have been elucidated. Zebularine is a 2-oxopyrimidine nucleoside with potent cytidine deaminase inhibitory activity. Zebularine is unique in that it is also endowed with good in vivo antitumor activity. This study attempts to identify the elusive electrophilic species that is formed during the decomposition of this drug and which could be responsible for its antitumor effect. The 5-fluoro analogue was also studied and found to behave similarly.

The synthesis of a series of conformationally constrained analogues of diacylglycerol was achieved. One of the many myristoylated 2-deoxypentonolactones synthesized was identified as equipotent to DAG in its capacity to compete for the phorbol receptor of protein kinase C (PK-C). This compound probably represents the conformation in which DAG binds to PK-C and hence this information could be of use for the design of either agonists or antagonists to PK-C. Some ether-containing analogues of the acyl-containing 2-deoxypentonolactones were synthesized. Complete biological evaluation is in progress.

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.

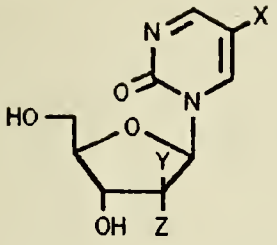
Pyrimidine-2-one Ribonucleosides. A Class of Cytidine Deaminase Inhibitors with Unusual Chemical Properties (Dr. Barchi, Dr. Marquez): The prototype 2-oxopyrimidine riboside (zebularine, 1) and the corresponding 5-fluoro analogue (2) are potent inhibitors of cytidine deaminase (CDA). In addition, among the known inhibitors of this enzyme, they represent a unique class of compounds since they are the only ones that simultaneously display significant in vivo L1210 antitumor activity. In order to better understand the mechanism of inhibition of CDA, our laboratory is collaborating with Professor Richard Wolfenden (Univ of North Carolina) by attempting the synthesis of a ^{13}C-labeled zebularine. Efforts to synthesize this compound have started employing labeled diethylmalonate as a starting material. Simultaneously, studies aimed at understanding the extreme lability of 1 and 2 to alkali have started with the purpose of discerning if there is a connection between this degradative process and the antitumor activity of the compounds. The final product of this degradative process for both compounds has been identified as the cyclic carbamate 4. When the corresponding ara-zebularine (3) was used, the alternate isomeric carbamate 5 was isolated. The first stable intermediate that forms during this degradation has also been characterized (structures 6 and 7). The nature of the residual fragment of the 2-oxopyrimidinone that is excised during this degradation is under investigation.

Synthesis of Protein Kinase C Inhibitors (Dr. Teng, Dr. Marquez): In this project the first goal has been to identify the conformation with which the natural agonist of PK-C (diacylglycerol, DAG, 8) binds to the regulatory site of the enzyme. During this year, all the isomeric 2-deoxypentonolactones representing all of the possible conformations of diacylglycerol (DAG) were synthesized. Myristic acid (tetradecanoic acid) was selected as the common fatty acid for these molecules. When these compounds were tested for their capacity to displace labeled phorbol ester from its binding site on PK-C, the most potent compound identified (9, K_i = 2.7 μM) had virtually the same K_i (1.3 μM) as glycerol-1-myristate-2-acetate (8). Thus, compound 9 represents a probable conformation with which the flexible agonist DAG (8) efficiently binds to PK-C. Since the role of the fatty acid ester is to give the molecule the proper buoyancy and orientation inside the membrane lipid bilayer, a series of derivatives of 9 with fatty acids ranging from 2 to 18 were synthesized. One important target was the compound with oleate (an 18-carbon fatty acid with a cis double bond). Biological tests are underway and lower K_i values are expected for the compounds with longer fatty acids. Once the most potent compound is found, it will be tested for its PK-C activating or inhibitory properties. The synthesis of the corresponding ether analogues, which are expected to behave as DAG antagonists, is in progress. Thus far, the ether derivative 10 has been synthesized and found also to be capable of displacing phorbol from its binding site, albeit with lower potency (K_i = 21.6 μM).

Publications:

Frick L, Yang C, Marquez VE, Wolfenden R: Binding of Pyrimidine-2-one Ribonucleoside by Cytidine Deaminase as the Transition-State Analogue 3,4-Dihydrouridine and the Contribution of the 4-Hydroxyl Group to Its Binding Affinity. Biochemistry 1989; 28: 9423-9430.

Goldstein BM, Bell JE, Marquez VE: Dehydrogenase Binding by Tiazofurin Anabolites. J Med Chem 1990; 33: 1123-1127.



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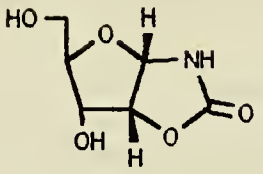

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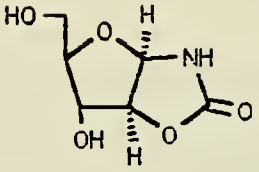

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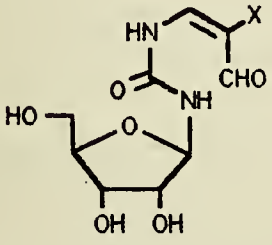

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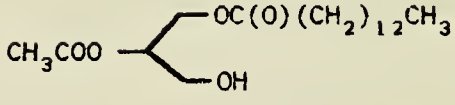

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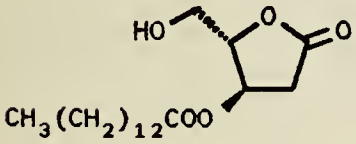

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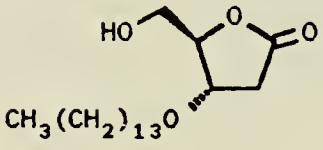


10

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06177-05 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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:
1.3	1.3	

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.)*

The objective of this project is the research and development of suitable analytical methods to: (I) establish the structure and purity of potential anti-AIDS agents and new antiviral drugs, (2) determine physical, chemical and biochemical 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. High-performance liquid chromatography and mass spectrometry are emphasized techniques. Compounds of current interest are dideoxynucleosides including ddG, 6-halo-dd-Purines, 2-NH_2-6-halo-dd-Purines, 2′-F-dd-ara-A, 2′-F-dd-ara-I, 2′-F-dd-ara-G, 2′-F-dd-C, 2′-F-dd-ara-C and 6-substituted-2′-F-dd-Purines.

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 biochemical, 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.

Major Findings:

1. Pharmacokinetics of 2′,3′-Dideoxyguanosine (ddG) in a Duck Model for Human Hepatitis B (Drs. Ford, Korenman, Mitsuya): Pekin ducks chronically infected with duck hepatitis B virus, a hepadnavirus containing reverse transcriptase, are a model for human hepatitis B. Intravenous bolus administration of ddG has been shown to decrease viral replication, as measured by DNA polymerase activity, in this duck model. In preparation for a study on the effects of long term administration of ddG on viral replication, the comparative pharmacokinetics and bioavailability of ddG administered subcutaneously at different sites were determined using a previously developed reverse phase HPLC method. Subcutaneous (s.c.) wing administration resulted in a greater than 85% bioavailability.

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 fluorine atom in the 2′-β-position of the sugar. 2′-F-dd-ara-C and ddC were equally stable in monkey plasma, exhibiting only 14% deamination in 28 hr. A group of 6-substituted purine dideoxynucleosides of this same series were evaluated as substrates for adenosine deaminase by determination of K_M, v_{max}, and relative rates of deamination *in vitro*. These dideoxynucleosides exhibited much slower enzymatic hydrolysis rates than either adenosine or ddA.

A rapid microscale method for determining partition coefficients by HPLC has been developed and evaluated. This micro-shake flask method requires only microgram quantities of compound and absolute sample purity is not essential. The partition coefficients of over 70 nucleoside analogs (log P range -2 to 1) have been measured using this microscale method. Good agreement (± 0.1 log P unit) has been observed with values determined by classical methods.

3. Analytical and Enzymatic Characterization of 6-Substituted Dideoxypurine Nucleosides as Potential Lipophilic Prodrugs with Anti-HIV Activity (Drs. Kelley, Ford, Mitsuya): The ability of a series of 6-halopurine dideoxyribofuranosides (6-halo-ddP) to inhibit the infectivity and cytopathic effect of HIV was shown to arise from their *in vitro* enzymatic conversion to either ddI or ddG. The ability of adenosine deaminase to hydrolyze 2-amino-6-halo-ddPs to ddG and 6-halo-ddPs to ddI was evaluated by the determination of the K_M, v_{max}, and

relative rate of *in vitro* deamination for each of these substrates. This enzymatic conversion to ddG was blocked in the presence of the adenosine deaminase inhibitor 2′-deoxycoformycin with the concomitant disappearance of anti-retroviral activity. In addition, all 2-amino-6-halo- and 6-halo-ddP nucleosides were much more lipophilic than either ddI or ddG, indicating enhanced potential for CNS penetration.

Publications:

1. 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 1989; 97: 1275-1280.

2. Marquez VE, Tseng CK-H, Mitsuya H, Aoki S, Kelley JA, Ford H Jr, Roth JS, Broder S, Johns DG, Driscoll JS. Acid-stable 2′-fluoro purine dideoxynucleosides as active agents against HIV. J Med Chem 1990; 33: 978-985.

3. Shirasaka T, Murakami K, Ford H Jr, Kelley JA, Yoshioka H, Kojima E, Aoki S, Driscoll JS, Broder S, Mitsuya H. Halogenated congeners of 2′,3′-dideoxypurine nucleosides active against HIV *in vitro*: a new class of lipophilic prodrugs. Proc Natl Acad Sci USA, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06178-05 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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:	Pamela L. Russ	Chemist	LMC, NCI
	Steven M. Musser	IRTA 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:
1.7	1.3	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 evaluated, 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, natural products and synthetic intermediates and products. Reductive dehalogenation of nucleosides during fast atom bombardment ionization has been studied and correlated with analyte and matrix electron affinity. 3-Nitrobenzyl alcohol has been evaluated as a liquid matrix for the fast atom bombardment analysis of natural products, nucleosides and acylated lactones. Micro-column liquid chromatography is under investigation for direct combination with a continuous-flow fast atom bombardment ionization interface.

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.

Major Findings:

1. Characterization and Inhibition of Reductive Dehalogenation During Fast Atom Bombardment (FAB) Mass Spectrometry of Substituted Nucleosides (Drs. Kelley, Musser): Reductive dehalogenation occurs during FAB ionization and leads to the production of positive or negative ions which appear to be derived from MH-X, where X is F, Cl, Br or I. This reaction complicates the analysis of halogenated nucleosides and their metabolites. Dehalogenation of nucleosides during FAB/MS was found to vary widely and to depend on several factors. Matrix additives, which are capable of forming free radicals in solution, potentiated dehalogenation. For substitution at a given position in a nucleoside, dehalogenation occurred in the order I > Br > Cl > F.

2. Synthetic and Collaborative Project Support (Drs. Kelley, Fales, Musser, Ms. Russ): The LMC continues to conduct an extensive program for the synthesis of a broad range of compounds of novel structure. Rapid and simple methods employing FAB mass spectrometry are routinely employed to characterize these new compounds without derivatization. Determination of elemental compositions from accurate mass measurement of FAB molecular ions has been utilized for structure confirmation. Negative ion FAB/MS techniques have been developed to characterize the choline and ethanolamine conjugates of 2′,3′-dideoxycytidine diphosphate.

3. Continuous-Flow Fast Atom Bombardment Mass Spectrometry with On-line Liquid Chromatography for Mixture Analysis (Drs. Kelley, Musser): Modifications to increase ion source pumping capacity have been made to our VG 7070E mass spectrometer and packed micro-column liquid chromatography systems are under investigation in preparation for implementing on-line liquid chromatography continuous-flow FAB/MS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06194-02 LMC

PERIOD COVERED: October 1, 1989 to September 30, 1990

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	Research Chemist	LMC, NCI
Other:	Marc Nicklaus	Visiting Fellow	LMC, NCI
	John S. Driscoll	Chief	LMC, NCI
	Victor E. Marquez	Research Chemist	LMC, NCI
	Terrence R. Burke, Jr.	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:
1.75	1.75	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.)*

Molecular modeling has been applied to a variety of problems in the LMC concerned with drug design and inhibition of binding of molecules to receptors.

Protein Kinase C. The role of diacyl glycerols as competitive inhibitors of phorbol for the active site of protein kinase C has been studied. The inhibitory effectiveness of such analogs as a function of their molecular shape can now be predicted fairly reliably.

Reverse Transcriptase Inhibitors. Modeling of nucleosides which inhibit reverse transcriptase has led to a definition of the molecular requirements for such inhibition.

Three-Dimensional Database. Use of the FCRF CRAY to develop this database has been abandoned and methods for accomplishing this work on a super mini-computer at NIH are under development.

Tyrosine Kinase. An extensive quantitative structure-activity relationship study of potential tyrosine kinase inhibitors has been completed.

Project Description:

General Objective:

The objective of this project is to provide, by means of molecular modeling 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. Design of tyrosine kinase inhibitors.

MAJOR FINDINGS

Design of inhibitors of protein kinase C

Studies of models of several esters related to diacyl glycerol and each of these molecules has been compared to phorbol. The results suggest that some of these should be competitive inhibitors of phorbol, while others should fail to compete. Such predictions have been generally accurate and have encouraged efforts to design new, more efficient phorbol inhibitors.

Design of compounds with anti-HIV activity

A very detailed study has been made of models of ddA, ddC and oxetanocin, all of which are known to be inhibitors of reverse transcriptase. This has allowed the design of fraudulent nucleosides which show inhibitory activity with respect to reverse transcriptase.

Design of inhibitors of tyrosine kinase

Computer models of a series of compounds related to the known tyrosine kinase C inhibitor, erbstatin, have been developed and used as the basis for a study of the quantitative structure-activity relationships operating in this system. The method used, comparative molecular field analysis, accurately predicts the inhibitory activities of a variety of numerous compounds that are known to interact with tyrosine kinase and work is now in progress both to improve activity by structural modification and to design new inhibitors of the enzyme system.

Development of a database of three-dimensional coordinates.

Plans for development of a database of three-dimensional atomic coordinates had to be changed when it became clear that access to the NCI CRAY computer at FCRF would not be possible. Efforts have been underway to convert two-dimensional data to three-dimensional coordinates on one or more super minicomputers on the NIH campus. It will be necessary to write software to accomplish this task and this work is in progress.

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 06198 01 LMC

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Inhibitors of tyrosine-specific protein kinases as anticancer agents.

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

Terrence R. Burke, Jr.		Research Chemist	LMC, NCI
Others:	V. E. Marquez	Deputy Chief	LMC, NCI
	B. B. Lim	IRTA Fellow	LMC, NCI

COOPERATING UNITS *(if any)*

LTVB, DCE, NCI

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	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.)*

Three approaches toward the inhibition of tyrosine-specific kinases are being investigated. In the first approach, opiates were found to inhibit epidermal growth factor receptor (EGFR) autophosphorylation at low concentrations. This was reversed at higher concentrations. (+)-Morphine 1, the analgesically inactive isomer of morphine, did not show this reversal of inhibition at higher concentrations. A series of phosphonate-containing structures 2 was prepared. None of these were active against p56lck kinase, and testing is presently continuing in the EGFR system. Finally, the known EGFR kinase inhibitor 6 was prepared and shown to inhibit p56lck autophosphorylation without inhibiting exogenous substrate phosphorylation. Erbstatin 7, a potent inhibitor of EGFR kinase, was shown to be inactive against p56lck. Analogs 8 and 9, which combine features of both 6 and erbstatin were inactive. Analogs are presently being prepared to study selective inhibition of p56lck autophosphorylation.

Project Description:

General Objective:

The objective of this project is to develop anticancer agents based on the inhibition of tyrosine-specific protein kinases.

Specific Objectives:

The development of inhibitors modeled on the following structural motifs: A) Opioid Analogs. B) Phosphonate-Containing Analogs. C) Polyhydroxy Aromatic Benzylidenes.

Major Findings:

Opioid-Based Inhibitors (Dr. Burke, Dr. Marquez):

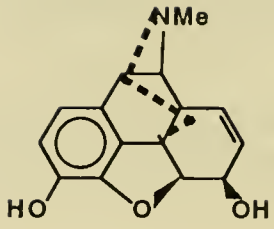
A total of 13 opiates were assayed for inhibition of autophosphorylation of epidermal growth factor receptor (EGFR) tyrosine kinase. Several of the opiates exhibited kinase inhibition at lower concentrations which was reversed at higher concentrations. (+)-Morphine 1, the analgesically inactive isomer of morphine, exhibited a more standard inhibition profile (increasing inhibition with increasing inhibitor concentration). The unusual profile of kinase inhibition is being studied further to examine its implications in enzyme function and design of inhibitors.

Phosphonate-Containing Inhibitors (Dr. Burke, Dr. Lim, Dr. Marquez):

A series of phosphonate containing benzylidenes 2 was prepared as well as naphthalene phosphonates 3 & 4. None of the compounds inhibited p56lck kinase activity. Testing in the EGFR system is in progress. Additional ring-constrained derivatives 5 are being prepared.

Polyhydroxylated Aromatic Inhibitors (Dr. Burke, Dr. Lim, Dr. Marquez):

Known 3,4-dihydroxy benzylidene 6 was shown to specifically inhibit autophosphorylation of p56lck without affecting phosphorylation of the exogenous substrate, rabbit muscle enolase. Erbstatin 7, a potent inhibitor of EGFR kinase, was found to be inactive in this assay. To examine the structural basis for the unusual activity of 6, analogs 8 and 9 were prepared by combining features of 6 and 7. Both 8 and 9 were inactive, indicating that combined features present in the ring and side chain of 6 are needed. A series of analogs is being prepared to further examine the structural requirements for selective inhibition of p56lck autophosphorylation.



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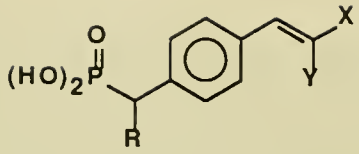

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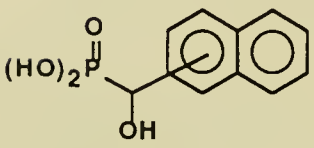
R = H, OH



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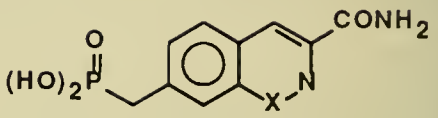

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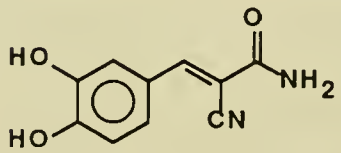

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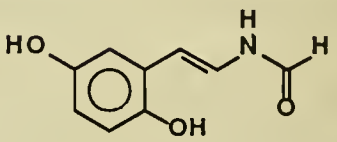

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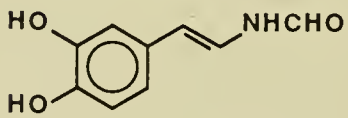

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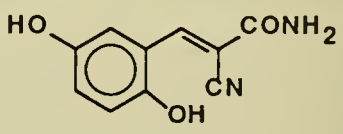

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06195-02 LMC

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Recognition Phenomena and Development of Inhibitors to HIV 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)*
Program Development Research Group, DTP, NCI
Laboratory of Molecular Virology and Carcinogenesis, ABL-Basic Research Program, NCI-FCRDC

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	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.)*

Peptides were synthesized that were expected to interact with specific structural and/or functional domains of the homo-dimeric 99 residue long HIV protease, and in this manner exert inhibitory activities. Design strategy utilized the generation of anti-sense peptides with optimized hydropathic anticomplementarity to the native sequences. For comparison the DNA based anti-sense peptides were also examined. Of 42 such synthetic peptides tested in the in vitro enzyme inhibition assays using recombinant HIV protease, 13 showed inhibitory activity in the IC50 range of 0.15-3.30 mM. Hydropathically optimized anti-sense peptides generated to the flap and to the alpha helical region of the enzyme were found to be the most effective inhibitors. Complementary peptides to the catalytic site region, encompassing the Asp-Thr-Gly triad, and to the C-terminal hinge region were comparatively less inhibitory. An anti-sense peptide, generated against the gag p17/p24 cleavage site was found to be active with an IC50 of 460 μM. One example also demonstrated that an all-D amino acid containing peptide can have inhibitory properties. Efforts are focussed in designing and evaluating more effective inhibitors, and to better understand the nature of intermolecular recognition phenomenon using physicochemical methods.

Project Description:

Objectives:

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 affecter molecules of receptor substrate complexes.

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. Structure based design of inhibitors is now possible, since the X-ray structure of the viral enzyme is available. Using the "sense/anti-sense" concept of molecular recognition phenomenon [Blalock and Smith (1984), and Fassina et al.(1989)], we designed and synthesized 42 peptides that were specifically targeted to bind to structural or functional domains of the enzyme. The targeted peptides were tested for enzyme inhibition using purified recombinant HIV protease.

In results to date 13 of the 42 peptides showed enzyme inhibitory activities in the IC50 range of 0.15-3.3 mM. The following enzymic structural regions were targeted:
1. Catalytic site region, encompassing the catalytic triad, Asp-Thr-Gly, (residues 25-27). Two of four 10-15 residue long peptides showed weak inhibitory activity, IC50 = 1.55 mM and 3.35 mM, respectively.
2. Flap region (residues 45-55). Of the 12 peptides tested, the best inhibitory activity was found with a hydropathically optimized 11-mer (IC50 = 155 μM). N-Terminal acetylation, C-terminal amidation, deletion at N-terminal, or expanding the chain length diminishes or abolishes the activity. The all-D configuration 11-mer peptide was approx. 3 times less inhibitory. The retro inverso analog was not inhibitory.
3. Alpha helical region (residues 85-94). Of 5 peptides tested 3 were found to be inhibitory. The best inhibitor showed an IC50 of 273 μM.
4. C-Terminal region (residues 90-99) encompasses both the subunit dimerization contact site (95-99) and part of the alpha helical region. Of 12 peptides tested, 2 showed some activity in the mmolar range only. Also, none of the 7 anti-sense peptides targeted to the N-terminal hinge region (1-10) was found to be inhibitory.
5. Gag p17/p24 cleavage site. One of 2 hydropathically optimized anti-sense peptides designed to bind to the substrate 9-mer, VSQNYPIVQ, was found to give an IC50 of 460 μM in the enzyme inhibition assay.

The approach utilized here allows for sequence specific targeting of peptidic agents to structural or functional domains of proteins. The results demonstrate that such design strategy can provide enzyme inhibitors. The agents reported here are of entirely different nature than the inhibitors generated by substrate analog based drug design. The current approach may provide all D configuration containing peptides as proteolytically stable inhibitors.

The hydropathic optimization approach provides better inhibitors than the anti-sense peptides obtainable on basis of the corresponding DNA sequence.

Publications:

1. 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. 1989; 264: 11252-11257.

2. Benjamin T, Niu C-H, Parmelee DC, Huggett AC, Yu B, Roller, P and Thorgeirsson SS. Direct N-terminal sequence analysis of rat liver plasma membrane glycoproteins separated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis, 1989; 10: 447-455.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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 further investigated in cultured Molt-4, CEM and ATH8 cells. Unlike 2′,3′-dideoxyadenosine (ddA), this fluorinated nucleoside is wholly resistant to decompostion 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 decompostion 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.

Studies of the cellular pharmacology of the dideoxynucleoside 2′,3′-dideoyxguanosine (ddG) have been initiated during the past year. This compound is of particular interest because of its activity against hepadnaviruses (notably hepatitis B), in addition to its activity in inhibiting the replication of HIV-1 and other retroviruses. In addition, ddG is a prototype for other anti-HIV dideoxynucleosides of potential clinical interest such as carbovir and 2,6-diaminopurine-2′,3′-dideoxynucleoside. In human T-cell lines in culture (Molt-4, CEM), the active metabolite ddGTP is generated in concentrations just adequate to inhibit retroviral reserve transcriptase. ddG appears to enter cells by simple diffusion, as determined by the inability of nitrobenzylthioinosine to impede its interiorization. Intracellularly, ddG is phosphorylated principally by the phosphotransferase activity of cytoplasmic 5′-nucleotidase, which preferentially utilizes IMP as a phosphate donor. Inhibitors of IMP dehydrogenase (e.g. ribavirin, tiazofurin, mycophenolate) increased the formation of ddG nucleotides by factors of from 10 to 40-fold. Marked enhancement of anti-HIV activity in the ATH8 assay system was also observed with these combinations, and particularly, with the combination ddG: ribavirin. This enhancement was accompanied by a parallel increase in IMP, strengthening the contention that phosphotransferase is crucially involved in the initial activation of ddG.

Studies of the clinical pharmacology of the anti-HIV nucleoside ddI have continued over the past year, in collaboration with Clinical Oncology Program, DCT, NCI. In human subjects, ddI has a plasma half-life of 38 minutes; bioavailability of the compound when administered orally together with oral antacids averaged 38%. CSF/plasma ratios for ddI (one-point determinations) averaged 21%; urinary recovery of unchanged parent drug was 36%. ddI thus has pharmacokinetic properties suitable for long-term administration to human subjects.

The cellular pharmacology of the antineoplastic nucleoside cyclopentenyl cytosine (CPEC) has been extensively investigated in Molt-4 lymphoblasts in vitro. CPEC is converted to nine metabolites in these cells: cyclopentenyl uridine (CPEU), CPEUMP, CPEUDP, CPEUTP, CPECMP, CPECDP and CPECTP (the active metabolite), together with two phosphodiesterase-sensitive nucleotides provisionally identified as CPECDP-choline and CPECDP-ethanolamine. CPECTP accumulated rapidly in these cells, ultimately reaching levels 100-200 times those of the parent drug in the medium; such levels of CPECTP can be measured by HPLC without recourse to radiolabel, a feature of value for the forthcoming clinical trials with CPEC.

Publications:

1. Ahluwalia, GS, Grem, JL, Hao, Z. and Cooney, DA. Metabolism and action of amino acid analog anti-cancer agents. Pharmac. Ther. 1990; 46: 243-271.

2. Hao, Z, Cooney, DA, Farquhar, D, Perno, CF, Zhang, R, Masood, K, Wilson, Y, Hartman, NR, Balzarini, J. and Johns, DG. Potent DNA chain termination activity and selective inhibition of human immunodeficiency virus reverse transcriptase by 2′,3′-dideoxyuridine-5′-triphosphate. Molecular Pharmacology, 1989; 37: 157-163.

3. Hartman, NR, Yarchoan, R, Pluda, JM, Thomas RV, Marczyk, KS, Broder, S. and Johns DG. Pharmacokinetics of 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine in patients with severe HIV infection. Clinical Pharmacology and Therapeutics, 1990; 47: 647-654.

4. Hartman, NR, Johns, DG. and Mitsuya, H. Pharmacokinetic analysis of dextran sulfate as pertains to its clinical usefulness for therapy of HIV infection. AIDS Research and Human Retroviruses. 1990; 6: 805-812.

5. Kang, GJ, Cooney, DA, Moyer, JD, Kelley, JA, Kim HY, Marquez, VE. and Johns DG. Cyclopentenylcytosine triphosphate: Formation and inhibiation of CTP synthetase. J. Biol. Chem. 1989; 264: 713-718.

6. Kensler, TW. and Cooney, DA. Inhibitors of the de novo pyrimidine pathway. In: Merton, S. Smith, HJ, eds. Design of Enzyme Inhibitors As Drugs. New York: Oxford University Press. 1989: 379-401.

7. Masood, R, Ahluwalia, GS, Cooney DA, Fridland, A, Marquez, VE, Driscoll, JS, Hao, Z, Mitsuya, H, Perno, CF, Broder, S. and Johns, DG. 2′-fluoro-2′,3′-dideoyxyarabinosyladenine: A metabolically stable analogue of the antiretroviral agent 2′,3′-dideoxyadenosine. Molecular Pharmacology. 1989; 37: 590-596.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06197-01 LBP

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Preclinical and Clinical Pharmacology of Anti-HIV Agents

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

P.I.:	N. Hartman	Sr. Staff Fellow	LBP, NCI
Others:	R. Yarchoan	Medical Officer	COP, NCI
	H. Mitsuya	Senior Investigator	COP, NCI
	J. Pluda	Sr. Medical Staff Fellow	COP, NCI
	R. Thomas	Protocol Nurse	COP, NCI
	K. Marczyk	Protocol Nurse	COP, NCI
	S. Broder	Director	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	

CHECK APPROPRIATE BOX(ES)

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

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

2′,3′-Dideoxyinosine (ddI) is in clinical trial as an anti-HIV agent. This unit is following the clinical pharmacology of this compound. Intravenously administered ddI has a half life of about 38 minutes. ddI is very acid labile; therefore for the phase I clinical trials the compound was given to fasting patients along with an oral antacid. The average oral bioavailability of this preparation was 38%. Other oral dosage forms were investigated to find a more convenient preparation and/or one with better bioavailability. CSF/plasma ratios and urinary excretion of ddI were measured also; CSF/plasma ratio averaged 21% for one point determinations, urinary recovery was 36%. It was determined that ddI had pharmacokinetic properties amenable to long term administration.

Dextran sulfate (DS) has also been investigated as an anti-HIV agent and has generally been given orally. There is little reason to believe that this compound will be absorbed orally, however. To evaluate this, we administered tritium-labeled DS to rats. When administered intravenously, the compound had a plasma half-life of about 30 minutes, and partially degraded DS could be retrieved in the urine. No intact DS could be seen in either plasma or urine when the compound was administered orally. Thus it is unlikely that this compound will be useful as an oral medication.

1. Studies with 2',3'-dideoxyinosine

ddI is one of a series of dideoxynucleosides investigated for anti-HIV activity in this institution. Initial studies used dideoxyadenosine (ddA); however, this compound was deaminated so rapidly that only ddI could be detected in the plasma of patients, even during continuous intravenous administration of ddA. For intravenous studies ddI was administered at doses starting at 0.2 mg/kg and progressing to 12.8 mg/kg in doubling escalations. The half life of ddI proved to be on the order of 38 minutes. This may not reflect the effective lifetime of the active species, however, because we have shown in earlier work that ddI is eventually activated to ddATP, which has an intracellular half-life of 8-24 hours. ddI was also found to have a total body clearance of ≈1/kg/hr, a renal clearance of .31 l/kg/hr, and a steady state volume of distribution of ≈1 l/kg.

Since ddI is extremely acid labile, there was some concern as to its oral bioavailability; therefore ddI was given as an oral solution to fasting patients along with an oral antacid. Using this protocol, an oral bioavailability of 38% was obtained. In an effort to improve the bioavailability as well as to make administration easier and compliance more likely, several other oral dosage forms were evaluated. Citrate buffered tablets had a low and erratic rate of absorption unless supplemental antacid was given, in which case it had an oral bioavailability equal to that of the standard solution. The absorption of two enteric coated preparations was on the order of that of the oral solution (26% and 36%), however the peak plasma level occurred at a much later time than the solution and was much lower. The preparation chosen for the phase II clinical trials was a citrate buffered solution packaged in a sachet for the patient to dissolve just before administration. This had an oral bioavailability of 29% in fasting patients, which decreased to 17% in the presence of food.

Since AIDS has a definite CNS component, the penetration of ddI across the blood-brain barrier needed to be established. For one point determinations, the CSF/plasma ratio of ddI averaged 21%. It is not known if the pharmacokinetics of ddI in the central nervous system parallel those seen in the blood, however.

The interactions of other medication on the pharmacokinetics of ddI were also investigated. Ranitidine, an H_2-receptor antagonist, would be expected to increase the bioavailability of ddI by inhibiting gastric acid secretion. When given to patients receiving the oral sachet, however, no consistent change in bioavailability was noted. Compounds which might interfere with the metabolism of ddI could also be expected to alter its pharmacokinetics. Ganciclovir, a guaninidine analogue effective against cytomegalovirus, is frequently given to AIDS patients and could interact pharmacokinetically with ddI. This compound did not produce any noticeable alterations in the pharmacokinetics of ddI in this study, however.

2. Studies with Dextran Sulfate

Dextran sulfate (DS) is one of a number of sulfated polysaccharides which can inhibit the destruction of HIV sensitive cells *in vitro*. The compound has also been administered orally in Japan for many years to treat hyperlipidemia, and seems to have few adverse effects. For these reasons a number of AIDS patients have been obtaining DS overseas and self-administering it orally. There is no reason to believe that the compound can be absorbed after oral administration, however;

therefore this lab has been examining the pharmacokinetics of DS in rats to determine if further human testing is warranted.

When rats received tritium-labeled DS as a bolus intravenous dose DS was eliminated from the plasma with an initial half-life of ≈30 min. The molecular weight of this compound (≈8000 MW) was unchanged during the first hour, but was degraded to material of about 4000 MW at two hours and beyond. DS of molecular weight >4000 retains most of the activity of the parent compound *in vitro*, however the activity drops rapidly after that and is not detectable for DS of MW <2000. Urine collected from these animals contained DS of molecular weight ≈4000, however less than 25% of administered material could be recovered in the urine. When DS was administered orally a small amount of radioactivity could be detected in the plasma which increased slowly with time, however it appeared to be only material completely degraded to tritiated water. No intact material could be detected in the urine of orally dosed animals either. It would therefore seem that the oral route would not be an effective means of administering DS.

Publications:

1. Hartman NR, Johns DG, Mitsuya H. Pharmacokinetic Analysis of Dextran Sulfate as Pertains to its Clinical Usefulness for Therapy of HIV Infection. AIDS Research and Human Retroviruses. 1990; 6: 805-12.

2. Hartman NR, Yarchoan R, Pluda JM, Thomas RV, Marczyk KS, Broder S, Johns DG. Pharmacokinetics of 2′,3′-Dideoxyadenosine and 2′,3′-Dideoxyinosine in Patients with Severe HIV Infection. Clinical Pharmacology and Therapeutics. 1990; 47: 647-54.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07181-05 LBP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Cellular Pharmacology of Chemotherapeutic Nucleosides

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

P.I.:	D. A. Cooney	Senior Investigator	LBP, NCI
Others:	E. Gregory	Biological Laboratory Technician (Animals)	LBP, NCI
	H. Ford	Biotechnical 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

Laboratory of Biochemical Pharmacology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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.)*

The cellular pharmacology of the antineoplastic nucleoside, cyclopentenyl cytosine, (CPEC), and of the antiretroviral nucleoside, dideoxyguanosine, (ddG), has been examined in cultured Molt-4 lymphoblasts. CPEC is converted to nine metabolites in these cells: cyclopentenyl uridine (CPEU), CPEU-MP, CPEU-DP and CPEU-TP; CPEC-MP, CPEC-DP and CPEC-TP, along with two phosphodiesterase-sensitive nucleotides provisionally identified as CPEC-DP-choline and CPEC-DP-ethanolamine. CPEC-TP accumulates rapidly in these cells, ultimately reaching levels 100-200 times those of parent drug in the medium; such levels of CPEC-TP can be measured by HPLC without recourse to radiolabel, a feature of value for the forthcoming clinical trials with CPEC. Molt-4 cells incubated with 5μM tritiated ddG, (97% of the label being in the sugar moiety) generate the active metabolite, ddGTP in concentrations just adequate to inhibit retroviral reverse transcriptase. The accumulation of ddGTP from ddG can be enhanced modestly by co-incubation with 2'-deoxyadenosine but is markedly stimulated by ribavirin, an antiviral agent in its own right. Tiazofurin and mycophenolic acid produce comparable stimulations, a feature which makes it likely that inhibition of IMP dehydrogenase, of which all these are potent inhibitors, is the common cause of their stimulatory effects on ddG anabolism.

1. Studies with Cyclopentenyl Cytosine, NSC 375575

Cyclopentenyl cytosine, CPEC, is a synthetic nucleoside containing the base cytosine engrafted onto the unsaturated carboxcyclic pseudosugar first found in the natural product neplanosine. CPEC is active in many animal tumor models by virtue of its inhibition of CTP synthetase. Phase I clinical trials with CPEC are expected to begin in the fall of 1990. Preparatory to those trials, we have examined the cellular pharmacology of CPEC in Molt-4 cells. The compound inhibits the growth of these cells with an IC_{50} of 50nM. Cytidine, even when added 24 hours after CPEC, restores a normal generation time, indicative of the fact that 1 day exposures to this agent do not kill these cells, but rather that the paucity of dCTP and CTP produced by the drug arrest cell growth by preventing DNA and RNA synthesis.

In short-term uptake experiments it was found that nitrobenzylthioinosine totally blocked cellular entry of CPEC, a finding which suggests that the compound is interiorized by the classical pyrimidine transporter which operates by facilitated diffusion. Inside the cell, CPEC undergoes anabolic and catabolic fates. The drug is converted to the expected 5′-mono, di and triphosphates (the last of which is the proximate antimetabolite in its capacity as a micromolar inhibitor of CTP synthetase) as well as to two phosphodiesterase-sensitive, phosphatase-resistant metabolites, which have been provisionally identified as CPEC-5′-diphosphocholine and CPEC-5′-diphosphoethanolamine. In addition, deamination of CPEC at some level occurs intracellularly as adjudged by the appearance of CPEU nucleotides and CPEU itself. Inasmuch as zebularine and 2′-deoxytetrahydrouridine totally abrogate the appearance of CPEU nucleotides whereas tetrahydrouridine does not, it is concluded that the deamination in question is likely to transpire at the level of the monophosphate, in a reaction catalyzed by 2′-deoxycytidylate deaminase.

It is noteworthy that preformed CPEU will, in molar excess, overcome the cytotoxicity of CPEC, so that its formation might serve to modulate the toxic and metabolic effects of its precursor.When the concentration of CPEC in the medium necessary to reduce CTP pools by 50% (an EC_{50}) was monitored over time by HPLC, this level was found to be ~400nM at 2 hrs, ~200nM at 4 hrs, ~100nM at 8 hrs and ~50nM at 24 hrs. These values correlated in an inverse way with the concentration of CPEC-TP measured in parallel extracts.

2. Studies with 2′,3′-Dideoxyguanosine (ddG)

Dideoxyguanosine is a dideoxynucleoside active in vitro against certain retroviruses (notably HIV-I) and hepadnaviruses (notably hepatitis B). The compound, while less potent in absolute molar terms than, for example, ddC, does exhibit a notably broad therapeutic index. Indeed, in a human marrow progenitor cell system, ddG was the "safest" of the dideoxynucleotides examined. During the present year we have investigated the cellular pharmacology of ddG using a preparation specifically (97%) labelled in the ribofuranose ring.

Using this compound we have demonstrated that ddG is not exceptionally cytotoxic to logarithmically growing Molt-4 cells (IC_{50}:150μM) nor does it exhibit

the delayed antiproliferativeefects of ddC. The drug appears to enter cells by simple diffusion as adjudged by the inability of nitrobenzylthioinosine to impede its interiorization. Intracellularly, ddG is phosphorylated, principally by the phosphotransferase activity of a cytoplasmic 5′-nucleotidase, which preferentially utilizes IMP as phosphate donor; deoxycytidine kinase also catalyzes a fraction of the initial phosporylation of the drug. Guanylate kinase then converts the resulting ddGMP to the level of the diphosphate, albeit at an efficiency that is two orders of magnitude lower than that exhibited by this enzyme with GMP as substrate. The activity responsible for the generation of ddGTP has yet to be definitively identified, but it is probably shared by several enzymes including adenylate kinase and purine nucleoside diphosphokinase.

During the past year, a search for agents able to augment the anabolism of ddG to ddGTP was mounted: it was found that deoxyadenosine functioned with ddG, as it had with ddA, as a modestly effective positive modulator of its phosphorylation. Of greatest interest however, is the observation that three classical inhibitors of IMP dehydrogenase - ribavirin, tiazofurin and mycophenolate - all increased the formation of ddG nucleotides by factors of from 10 to 40 fold. The fact that this enhancement was accompanied by a parallel increase in IMP - which as was mentioned - is a favored phosphate donor in the reaction catalyzed by the cytoplasmic phosphotransferase, strengthens the contention that this enzyme is crucially involved in the initial activation of ddG.

Publications:

1. Ahluwalia GS, Grem JL, Hao Z and Cooney DA. Metabolism and action of amino acid analog anti-cancer agents. Pharmac. Ther. 1990; 46: 243-271.

2. Hao Z, Cooney DA, Farquhar D, Perno CF, Zhang R, Masood K, Wilson Y, Hartman NR, Balzarini J and Johns DG. Potent DNA Chain Termination Activity and Selective Inhibition of Human Immunodeficiency Virus Reverse Transcriptase by 2′,3′-Dideoxyuridine-5′-triphosphate. Molecular Pharmacology, 1989; 37: 157-163.

3. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim HY, Marquez VE and Johns DG. Cyclopentenylcytosine triphosphate: Formation and inhibition of CTP synthetase. J. Biol. Chem. 1989; 264: 713-718.

4. Kensler TW, and Cooney DA. Inhibitors of the de novo Pyrimidine Pathway. In: Merton S and Smith HJ, eds. Design of Enzyme Inhibitors As Drugs. New York: Oxford University Press, 1989: 379-401.

5. Masood R, Ahluwalia GS, Cooney DA, Fridland A, Marquez VE, Driscoll JS, Hao Z, Mitsuya H, Perno CF, Broder S, and Johns DG. 2′-Fluoro-2′,3′-dideoxyarabinosyladenine: A Metabolically Stable Analogue of the Antiretroviral Agent 2′,3′-dideoxyadenosine. Molecular Pharmacology. 1989; 37: 590-596.

ANNUAL REPORT OF THE DRUG SYNTHESIS & CHEMISTRY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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. During this year, approximately 7,000 new compounds, (280 pure natural products and 6720 synthetic compounds) were acquired from 677 suppliers. The DS&CB plays a key role in operation of the Acquisition Input Committee, which manages the flow of compounds through both the AIDS and cancer screens. About 200 pure compounds weekly are being submitted to the AIDS screen. Active leads discovered during this period include polyoxymetalates (NSC 622102), diaryl sulfones (NSC D624231 and D627708), thiazolobenzimidazoles (NSC D625487), and a new oxathiin carboxanilide (NSC D629243). A tracking system of 12 check points was 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 submissions. During the restructuring phase of the anticancer program, approximately 8,000 compounds for cancer screening were acquired. These compounds are being screened in the <u>in vitro</u> cancer screen at the rate of 300 compounds per week. Identification of structure-activity relationships by computer-assisted analysis of test results is well-established within DS&CB. Active leads are optimized through the synthesis of prodrugs and congeners. Current efforts include the synthesis of ellipticinium analogs, castanospermine analogs, diazo dyes, active ATA fractions, novel platinum analogs, and bombesin antagonists. Other projects include the resynthesis of compounds unavailable from the literature, scale-up synthesis of lead compounds, and radiolabelled synthesis. A total of 14 radiolabelled compounds was synthesized during the period, including oxathiin carboxanilide (NSC 615985), and the ellipticinium acetate (NSC 627505). DS&CB also collaborates with DTP intramural scientists by synthesis of compounds for special projects; current examples are a series of anticancer N-mustards and CoQ analogs. We also support AIDS and cancer research by supplying compounds available in our repository for experimental purposes. This year, approximately 2,800 samples were shipped to investigators world-wide.

Over the past year, DS&CB has assumed new responsibilities for acquisition of natural products. We are in the process of initiating a contract effort for the synthesis of natural product analogs for structure-activity optimization. We have also been able to initiate a collaborative program with the Indian Institute of Chemical Technology for the synthesis of natural product analogs at no cost to us. Projects under way include the synthesis of sulfoglycolipid and a semi-synthetic approach to taxol synthesis.

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 90

Contractor	Investigator	Contract No.
Alabama, University of	Baker	N01-CM-87267
ERCI Facilities Service Corp.	Groover	N01-CM-07317
Georgia Technology Res. Center	Zalkow	N01-CM-87269
Illinois, University of	Farnsworth	N01-CM-87226
Japanese Foundation	Tsukagoshi	N01-CM-36011
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-97561
Research Triangle Institute	Kepler	N01-CM-87227
Southern Research Institute	Temple	N01-CM-87229
Starks Associates, Inc.	Risbood	N01-CM-87231
Starks Associates, Inc.	Robeson	N01-CM-97569
Z, Inc.	Sobers	N01-CM-73720
Master Agreements (Task Order) Contracts:		
Alabama, University of	Baker	N01-CM-07335
H.G. Pars Pharmaceutical Labs.	Pars	N01-CM-07339
Research Triangle Institute	Seltzman	N01-CM-07330
Ricerca, Inc.	Buchman	N01-CM-07331
Southern Research Institute	Temple	N01-CM-07329
SRI International	Tanga	N01-CM-07333
Starks Associates	Hsiao	N01-CM-07341

PUBLICATIONS:

Michalak RS, Myers DR, Parsons JL, Risbood PA, Haugwitz RD, Narayanan VL. The synthesis of Irisquinone, Tetrahedron Lett 1989;30:4783-6.

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 1989;81:1088-92.

Narayanan VL, Nasr M, Paull KD. Computer assisted structure-antileukemic activity correlations of organotin compounds and initial exploration of their potential anti-HIV activity, NATO ASI Ser Ser H 1990;37:201-17.

Haugwitz RD, Anderson WK, Plowman J, Kaslinal R, Houston DM, Narayanan VL. [Bis(aminomethyl)dimethylsilane]platinum(II) dichloride: a potential antitumour agent, John Wiley & Sons Ltd. In press.

Chapter in a Book

Hodes L. Computer-aided selection for large-scale screening. In: Kennewell PD, ed. Comprehensive Medicinal Chemistry, Vol. 1, general introduction. Oxford: Pergamon Press, 1990;279-84.

Narayanan VL, Nasr M, Paull KD. Computer-assisted structure - Anti-leukemic activity analysis of purines and their aza and deaza analogs, Basel: Prog Drug Res 1990;34:319-41.

PATENTS:

Baker DC, Hand ES, Haugwitz RD, Narayanan VL, Rampal JB. US Patent 4,709,033: Pyrazine Diazohydroxide Compounds and Methods for their Production and Use, November 24, 1987.

Narayanan VL, Baker DC, Haugwitz RD, et al. US Patent 4,845,101: Pyrazine Diazohydroxide Compositions and Method of Use as Cytostatic Agent; Antitumor, Leukemia Treatment, July 4, 1989.

ANNUAL REPORT OF THE NATURAL PRODUCTS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 - September 30, 1990

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 National Cancer Institute (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 Developmental Therapeutics Program (DTP) screening programs; (2) coordination of research directed toward isolation of new agents from active extracts; and (3) large-scale procurement of new agents for preclinical and clinical development. 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 marine protozoa was awarded in March, 1989, and a project for the cultivation of unusual fungi was initiated at the Frederick Cancer Research and Development Center (FCRDC) in March, 1990. An initiative for the cultivation of marine anaerobic bacteria is due to be implemented in the near future.

An important aspect of the Branch's efforts continues to be 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 16,000 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. Several novel classes of in vitro active products have been isolated from plant extracts by the DTP intramural isolations chemistry group, and work is proceeding on the fractionation and purification of other active extracts from plant, marine organism and microbial sources. A similar program is now in operation for the discovery of novel anticancer agents from natural sources, and over 5,000 extracts have been submitted for testing.

Organization and Staffing

The Branch is organized into four functional segments which are: (1) discovery of new agents from fermentation derived extracts; (2) discovery of new agents from plant extracts; (3) discovery of new agents from extracts of marine

organisms; and (4) large-scale isolation and purification of new agents for preclinical and clinical development. 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 two secretaries. The contracts managed by the Branch are outlined in Table I.

Table 1.

Natural Products Branch Contracts

Contractor	Investigator	Contract No.	Contract Program Area
		Cancer	
Australian Institute of Marine Science	Murphy	NCI-CM9-7597	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 (Latin America)
Univ. of Hawaii at Manoa	Patterson	NCI-CM6-7745	Cultivation of Cyanobacteria
Martek Corporation	Behrens	NCI-CM9-7615	Cultivation of Marine Protozoa
		AIDS	
Univ. of Hawaii at Manoa	Sagawa	NCI-CM8-7282	Plant Recollections
World Botanical Associates	Spjut	NCI-CM8-7281	Plant Recollections
Univ. of Illinois at Chicago	Soejarto	NCI-CM0-7345	Plant Recollections

Missouri Botanical Garden	Forero	NCI-CM0-7346	Plant Recollections
New York Botanical Garden	Balick/Daly	NCI-CM0-7347	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	Hoeksema	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 the University of Hawaii have exhibited preliminary in vitro AIDS antiviral activity. A novel class of compounds exhibiting in vitro anti-HIV activity, the glycosulfolipids, has been isolated from several cyanobacterial extracts; these cyanobacteria are now being cultivated on a large scale in order to obtain sufficient material for further development. A project for cultivation of a select group of fungi belonging primarily to the class, Zygomycetes, has been initiated with the contractor at FCRDC. In addition, plant materials being received from the NCI collection contractors operating in tropical regions worldwide, are being investigated as a possible source of unusual fungi. An initiative for the cultivation of marine anaerobic bacteria will soon be implemented.

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 18,000 samples have been received of which over 8,200 have been extracted to yield over 16,400 extracts.

Master Agreement Contracts for the recollection of plants for the isolation of potential AIDS antiviral agents have been awarded to five organizations listed in Table I.

Marine Program

A major contract was initiated September, 1986 for the collection and documentation of 5,000 shallow-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. This contract is meeting its goal of more than 1,000 specimens per year and over 3,500 are on hand in the FCRDC Repository. Over 1,300 extracts have been prepared according to protocols developed at FCRDC. A contract for the collection of deep-water organisms was also awarded in September, 1986, but was terminated after one year due to escalating costs, difficulty in scheduling ship time, and insufficient amounts of samples collected. With the improvement of less expensive ROV (remotely operated vehicle) technology, NCI might consider restarting this project at a later date, since evidence is mounting that deep-water organisms might yield novel bioactive agents of interest to the program.

Natural Products Extraction Laboratory and Repository

A natural products extraction laboratory and repository is operating at the Frederick Cancer Research and Development Center 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^{0}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 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, 1990

	Organisms Received	Organisms Extracted	Extracts Available	Extracts Tested for Anti-HIV Activity
Marine Invertebrates	3,535	650	1,313	924
Cyanobacteria	270	270	540	540
Fungi	1,103	1,103	12,397	3,635
Terrestrial Plants	18,446	8,221	16,442	9,639
Marine Plants	778	383	766	507
Lichens	367	353	706	691

Accomplishments

The major program for the discovery of new leads for development into anticancer and AIDS antiviral agents continues to progress well. Nearly 16,000 extracts of plant, marine and microbial origin have been submitted for testing in the in vitro anti-HIV screen, and over 1,000 have shown preliminary activity. Of these, over 200 are being actively investigated with a view to isolation of the pure active agents. A new class of in vitro active agents, glycosulfolipids, has been isolated from several cyanobacterial extracts, and these compounds are being studied further. Several new classes of in vitro active anti-HIV agents have been isolated from plants. Over 5,500 extracts have been submitted for testing in the human cancer cell line screen, and results are currently being analyzed. Productive collaborations have been established with a number of research groups in the study of medicinal plants. These include Dr. Paul Cox of Brigham Young University (Samoan Plants), Dr. Won Sick Woo of Seoul National University, and scientists of Kunming Botanical Research Institute in the People's Republic of China.

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 New Delhi, the American Society of Pharmacognosy meeting in San Juan, Puerto Rico, and a Ciba Foundation Symposium on "Bioactive Compounds from Plants" held in Bangkok.

Publications:

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Beutler JA, Alvarado AB, McCloud TG, and Cragg GM. Distribution of phorbol ester bioactivity in the Euphorbiaceae, Phytother Res 1989;3:188-192.

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ANNUAL REPORT OF THE BIOLOGICAL TESTING BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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 FCRDC, 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

A. Replace where indicated current human tumor panel lines with well-characterized and defined lines which include a documented patient clinical history.

B. To expand in vitro screening capacity from the current level to our goal of 20,000 compounds on an annualized basis.

C. Develop secondary in vitro testing capabilities for follow-up studies with agents of interest.

D. Develop special testing capabilities for important types of cancer, e.g. prostate and breast that are not amenable for testing under current in vitro screening protocol.

E. Continue to develop cell lines when current lines are not available or are considered to be doubtful regarding representative value for correlating human cancer types.

F. Continue to develop in vivo models, e.g. orthotopic that have the potential for more accurately predicting clinical activity.

G. Provide "customized" in vivo testing capabilities to pursue all leads from the in vitro screen efficiently.

II. AIDS Area

A. To maintain in vitro screening capacity at an annualized level exceeding 50,000 tests.

B. To enhance secondary in vitro testing capabilities for follow-up studies with agents of interest from the initial screen.

C. To develop and utilize in vivo models that are likely to be more representative of the clinical disease.

D. To perform in vivo studies with these models utilizing agents of interest from primary and secondary in vitro testing.

III. Animal Production and Tumor Procurement

A. 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.

B. To obtain a sufficient supply of human tumor cell line materials and xenografts, and to develop this material through extramural and FCRDC 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

A. Initial In vitro screen

Screen has reached annualized level of 15,000 tests with expansion to 20,000 expected very soon.

B. Secondary and specialized testing

1. In vitro

(a) Protocols for confirmatory testing have been developed. Protocol selection is based on tumor type and desired information.

(b) Studies are in progress to evaluate protocols and initiate testing with prostate cells. It is considered possible/probable that protocols may be

utilized for studying breast and other types of cancer.

2. In vivo

(a) Testing has been performed as indicated with a limited number of agents from the in vitro screen. Primarily, testing has been performed subcutaneously with appropriate correlating lines.

(b) Approximately 90% of the current in vitro testing panel lines are passaged in vivo on a continuing basis and available for testing.

(c) EORTC agreements are in place for in vivo studies as indicated to supplement and confirm contractural capabilities in the USA.

II. AIDS Area

A. In vitro screen

1. The AIDS in vitro screen is now operational at a level exceeding 40,000 tests annuallly. It is anticipated that this screen will operate at a 50,000 annualized test level in the short term.

2. Confirmatory testing of active agents place between contractor laboratories and with other systems including a syncitium assay and the production of HIV p24 antigen.

B. In vivo testing

1. Currently available in vivo AIDS models have limited applicability regarding the disease in humans. Model development studies are in progress. Candidate models include a closely related lentivirus (BIV) and the SCID/NIH III mouse carrying HIV.

III. Animal Production and Tumor Distribution

A. 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.

B. 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 for the cancer testing program and

to make these lines available for distribution. The payback system for cell line distribution is working successfully.

BIOLOGICAL TESTING BRANCH
FY 1990

PRIMARY RODENT CENTERS (3)	$3,530,319
Supply breeding nucleus for the animal program and athymic mice for drug evaluation.	
RODENT PRODUCTION CENTERS (1)	216,000
Large-scale production of nuce mice under barrier controlled environment.	
DIAGNOSTIC & HISTOCOMPATIBILITY PROJECTS (7)	635,643
To monitor animal health and genetic integrity.	
DEVELOPMENT OF STANDARDS & GUIDELINES (1)	44,500
For animal care and breeding.	
QUALITY CONTROL AND MODEL DEVELOPMENT (1)	1,028,598
PRIMARY SCREENING OF HUMAN AIDS VIRUS	692,173
HUMAN TUMOR CELL LINE PROCUREMENT	
Breast (1)	166,199
Prostate (2)	551,428
FREDERICK CANCER RESEARCH AND DEVELOPMENT CENTER (2)	
Animal Production	3,576,627
CANCER RESEARCH AND SUPPORT PROJECTS	7,101,425
AIDS RESEARCH AND SUPPORT PROJECTS	4,841,158
TOTAL ESTIMATED COST	$22,384,070

The above figures do not reflect reimbursements from grantees, VRB, and other NCI Divisions that are credited to the contracts.

ANNUAL REPORT OF THE INFORMATION TECHNOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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.

Last year, the computing systems of DTP underwent major revisions in both a hardware and database design. Emphasis this year was on the development of new or revised software systems. Also, there were important new additions to the ITB staff and there were important changes among the applications contractors. Despite the many changes last year and this, it has become clear that many more changes in hardware and software will be required in the coming year as well.

The new software systems developed this year are discussed briefly below.

A new cancer supplier report package was developed. The package itself consists of four pages: The Data Sheet - A page listing the relevant mean optical densities for the test wells (at five doses), the control wells, and the time zero wells. Also listed are the Percentage Growth values calculated for each test well and three special reference concentrations, the GI50, the TGI, and the LC50; The Dose Response Curves - A curve plotting the Percentage Growth vs the concentration is presented for each cell line. These curves are grouped according to subpanel (Leukemia, Non Small Cell Lung Cancer, Ovarian, etc); The Mean Graphs - Three mean graphs are presented - one depicting activity at the growth inhibitory GI50 level, one depicting activity at the cytostatic TGI level, and one at the cytotoxic LC50 level; The Dose Response Matrix - A new graphic, the Dose Response Matrix appears together with a battery of statistics which quantify the degree of subpanel selectivity demonstrated by the compound tested. Also, the statistics are used to select the most selective-appearing mean graph from among the three on the Mean Graph page. This one is reprinted next to the Dose Response Matrix. The statistics appearing on the Dose Response Matrix, the mean $\log_{10}$ GI50 and Delta from the Mean Graphs are all collected in an Oracle table and used as the basis for a triage system. This triage system permits the separation of the compounds tested into two categories: one category which requires committee

review to determine screening program interest and one category of non-selective, non-cytotoxic compounds for which no further work is anticipated. A tracking system was developed to manage the supplier reporting in cancer. An on-line system, called the CSRS was developed and made widely available such that any user can order cancer supplier reports for NSC numbers that interest them.

An AIDS Decision Module was developed which assists the antiviral evaluation group in their efforts to document and track antiviral testing of compounds. The antiviral tests on a given compound are performed serially over several weeks and this program brings the individual test results together and generates a conclusion for the serial results. Several modifications were made to the AIDS supplier report with guidance from the antiviral evaluation group. New subsystems to support laboratory data collection for new AIDS-related tests were developed. In particular, software was developed to support the syncytial assay and the P24 assay. Plate analysis reports (PARS) became available from the VAX to eliminate the need for the daily mailing of laboratory generated PARS. A new VAX version of a AIDS compound tracking systems was developed to replace an earlier system on the IBM 3090. This program provides tracking from date of acquisition to time supplier reports are mailed out. An on-line system, called the ASRS, was developed and made widely available such that any user can order AIDS supplier reports for NSC numbers that interest them.

New versions of the Assign/Avail subsystems were developed for AIDS and cancer. The previous systems were highly troublesome, accounting for the majority of complaints received about software problems. The new systems are totally redesigned and incorporate excellent plate tracking capabilities not heretofore available. These two programs were each the product of the new applications contracts awarded to CTIS (one for cancer one for AIDS).

It became necessary to develop an entirely new computer system to record <u>in vivo</u> screening data. Two previously developed systems, one from Dr. Feibig and one from Southern Research Institute provided a basis from which to initiate the new system. The new system uses Oracle tables as the main database system, SQL Forms for input screens, and graphic outputs are created through SAS.

As part of a continuing effort to make the DTP computer system more accessible for the average user, work began on a menu system (called the User Friendly Interface, UFI) which, when fully developed, will list all available programs in the DTP system and will permit the user to select and execute their selection from among them with a few keystrokes. On-line context-sensitive help will be available, replacing the old instruction manual system.

Publications

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Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Waigro-Wolff A, Gray-Goodrich M, Campbell H, Boyd M. Feasibility of a High-Flux Anticancer Drug Screen Utilizing a Diverse Panel of Human Tumor Cell Lines in Culture, J Natl Cancer Inst. In Press

Chapter in a Book

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Boyd MR, Paull KD, Rubinstein, LR: Data Display and Analysis Strategies for the NCI Disease-Oriented In Vitro Antitumor Drug Screen. In: Baker L, et al, eds. Proceedings of the 22nd Annual Detroit Oncology Symposium on Anticancer Drug Discovery and Development, Detroit:1990. In Press.

ANNUAL REPORT OF THE GRANTS AND CONTRACTS OPERATIONS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

This Branch provides an administrative and managerial focal point for the support and coordination of DTP extramural activities, including grants, contracts and cooperative agreements. Activities for this year are summarized below by funding mechanism.

Grants

Grants are an assistance mechanism used when (1) the idea for the project is initiated by the investigator; (2) no substantial program involvement is anticipated between the NCI and the recipient during performance of the activity, thus allowing the recipient freedom of action in carrying out the research project; and (3) there is no expectation on the part of the NCI of a specified service or product. The Biochemistry and Pharmacology Grant Program comprises all aspects of anticancer drug discovery and development research. A breakdown by topic subcategory is shown in Table 1. At the end of FY 1989, 332 grants totaling $52,784,606 were administered by the Branch. During FY 1990 a total of 385 grant applications were received. About 25% are expected to be awarded by the end of the fiscal year. During the past year new program referral guidelines were implemented to reflect current research priorities and to incorporate recent advances in modern drug design and development, such as computer modeling and newer techniques from molecular biology and biotechnology. 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.

Although there were several important research findings during the past year, three grantees made outstanding contributions. Dr. Stephen Lippard, a Merit Awardee from the Massachusetts Institute of Technology (CA 34992), led a team which succeeded in the isolation of cDNAs modified by the anticancer drug cisplatin. The discovery of this specific protein may shed light on the way tumor cells repair cisplatin-induced DNA damage. Dr. Igor Roninson of the University of Illinois at Chicago (CA 40333) developed more sensitive methods to measure multidrug resistance (MDR) using the polymerase chain reaction and subsequently made the important observation that MDR expression varies among different human tumor types, and sometimes is detectable only by PCR. Finally, Dr. Monroe Wall (CA 38996) improved the synthesis for 9-amino-20(RS)-camptothecin, an agent selected for development to clinical trial by the NCI. The compound, which is active against several murine and human tumor models, has poor water solubility, but a number of formulation approaches appear promising. Recently, Dr. Wall has synthesized the natural (20S) and unnatural (20R) forms of the agent and found, in collaborative studies with Drs. Kurt Kohn and Yves Pommier, Laboratory of Molecular Pharmacology, DTP that the natural form is a much more potent inhibitor of topoisomerase I.

Contracts

DTP uses the contract mechanism to procure needed resources or services. In contrast to assistance mechanisms, which are used to support and stimulate research, contracts are used when the principal purpose of the procurement is to acquire a specific service or end product for the direct benefit of or use by DTP. The Branch coordinates all phases of the DTP contract process from the point of development of the concept descriptions for new and recompeting projects through award by the NCI Research Contracts Branch and the subsequent annual technical merit reviews and incremental fundings. The extramural contract program supports both the Cancer and AIDS drug discovery and development efforts, and extends to the pharmaceutical and chemical industries, universities, medical centers and private research organizations in the United States and abroad. The Branch functions as the source of guidance to DTP professional staff on all program-related aspects of research and development contract procurement and operations. The number of ongoing contracts exceeds one hundred.

Table 2 identifies by title the solicitations and awards for FY 1990. The estimated total value of the 1990 awards over the anticipated period of each contract is $19,093,972. Requests for Proposals for 14 projects were issued during FY 1990, for FY 1991 award, at an estimated cost of $41,820,852 for the total contract period.

Cooperative Agreements

Cooperative agreements are an assistance mechanism used when (1) the applicant is responding to a specific NCI Request for Application (RFA), (2) the approach to achieving the goals set forth in the RFA is initiated by the investigator, and (3) substantial program involvement is anticipated between the NCI and the recipient during the planning and performance of the activities. DTP has used this mechanism successfully since 1983 for implementing the National Cooperative Drug Discovery Group Program, an effort which exploits recent 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 research efforts. Currently there are eleven funded Groups (Table 3) at an FY 1989 cost of $5,447,816. During 1990, twelve more Groups have been approved for funding at an annual cost of about $9,000,000 (Table 4). The new awards will bring the total outlay to about $15,000,000 and will greatly expand efforts to discover new anticancer agents from natural sources, such as tropical rain forests and marine habitats, and will stimulate diverse types of research projects to find new therapies using three general approaches: general mechanism of action, specific disease-oriented strategy, and novel model development.

The NCDDG Program has been successful in bringing several new therapies to clinical trial. Four agents reported for the first time last year are still under active clinical investigation through other support mechanisms. These include Hycamptamine, an analog of camptothecin with improved water solubility which shows activity against several murine leukemic and solid tumor models and forms a complex with topoisomerase I and DNA; HomoDES, a potent inhibitor of polyamine biosynthesis which is in Phase I clinical trials at the Univer-

sity of Florida, Gainesville, FL; a monoclonal antibody called 225 IgG1 which binds with high affinity to the human EGF receptor and blocks EGF-induced activation of tyrosine kinase and which has been radiolabeled for ongoing human physiological distribution studies; and a novel diphtheria toxin-related interleukin-2 fusion protein known as DAB486-IL-2 which is being examined in patients with T-cell leukemias or lymphomas bearing high affinity interleukin-2 receptors. During this past year another product from the NCDDG Program has entered clinical trial. An anti-transferrin receptor antibody (42/6) is being used to treat patients with hematological malignancies. This protocol marks the first human trial using murine IgA antibody therapy.

Workshops

Last year the Branch cooperated with the Biological Response Modifiers Program (BRMP) in sponsoring a workshop entitled, "Combining Biological Response Modifiers with Cytotoxics in the Treatment of Cancer: Developing a Rational Approach to a New Therapy." The workshop, which was held March 5-7, 1990 in Baltimore, MD, provided an excellent forum for investigators to summarize their findings. The interactions of basic scientists and clinicians are likely to lead to new protocols for clinical trial. The Branch organized a workshop on "Taxol and Taxus: Current and Future Perspectives", which was held June 26, 1990 in Bethesda, MD. As a result of this workshop, an RFA (Request for Applications) will be issued to encourage further work on this clinically active antitumor agent and to stimulate novel approaches to solving the supply problem.

Publications

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2. Suffness M. Development of antitumor natural products at the National Cancer Institute. In: Takeuchi T, ed. Gann monograph no. 36. Antitumor natural products - basic and clinical research in memory of Professor Hamao Umezawa. Tokyo: Japan Scientific Societies Press, 1989; 21-44.

3. Suffness M, Pezzuto JM. Assays related to cancer drug discovery. In: Hostettmann K, ed. Methods in plant biochemistry - biological techniques. London: Academic Press, 1990; in press.

TABLE 1

BIOCHEMISTRY AND PHARMACOLOGY GRANTS

PROGRAM AWARDS BY SUB-CATEGORY

FISCAL YEAR 1989

A	Synthesis and Chemistry	89	$12,850,685
B	Natural Products	38	5,455,313
C	Screening and Experimental Therapeutics	46	6,114,349
D	Comparative Pharmacology	35	3,818,263
E	Other Preclinical Aspects	3	375,321
F	Mechanism of Action	118	19,724,770
	Program Projects	3	4,445,905
	Total	332	$52,784,606

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

Master Agreements for Chemical Synthesis	$ 1,418,625
Partial Support of Institute of Laboratory Animal Resources	237,500
Detailed Drug Evaluation and Development of Treatment Strategies for Chemotherapeutic Agents	4,248,144
Collection of Shallow Water Marine Organisms	1,059,317
Storage and Distribution of Clinical Drugs	3,721,411
Synthesis of Congeners and Prodrugs	4,144,224
Maintenance of a Rodent Production Center	775,515
Operation of an Animal Diagnostic Laboratory	1,381,407
Synthesis of Bulk Chemicals and Drugs for Preclinical and Clinical Studies	5,478,664
Synthesis of Bulk Chemicals and Drugs for Preclinical and Clinical Studies for Small Business	5,478,664
Master Agreements for Mechanism of Action and Biochemical Pharmacology Studies of Antitumor Agents	4,144,224
Total	$32,087,695

ANTI-AIDS PROJECTS

Preparation of Radiolabeled Anti-AIDS Compounds	2,364,375
Preclinical Pharmacology Studies of Anti-AIDS Agents	2,868,775
Large Scale Preparation of Anti-AIDS Bulk Drugs for Phase II and III Clinical Trials	2,657,276
Synthesis of Congeners and Prodrugs of Anti-AIDS Compounds	2,364,375
Development and Production of Parenteral Dosage Forms of Anti-AIDS Agents	3,039,097
Analysis of Chemicals and Pharmaceutical Formulations of Anti-AIDS Agents	2,237,881
Total	$15,537,779

ANTITUMOR AND ANTI-AIDS PROJECTS

Operation and Maintenance of DTP Biological Data Processing System	$ 2,895,152
Storage and Distribution of Chemicals and Drugs Used in Preclinical Evaluation and Development	3,830,706
Operational Systems Development in Support of the DTP	1,683,117

TABLE 2, CONTINUED

Natural Products Lead - Based Synthesis	2,364,375
Resynthesis of Compounds for Screening	2,522,000
Total	$13,295,350
GRAND TOTAL	$60,914,824

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	$ 391,398
1 U01 CA 50743-01	Chang, Ching-Jer Purdue University Mechanism-Based Discovery of Anti-tumor Agents	$ 361,207
1 U01 CA 50750-01	Clardy, Jon C. Cornell University New Anticancer Drugs From Cultured Marine Organisms	$ 428,587
5 U01 CA 45962-03	Corbett, Thomas H. Wayne State University Drug Discovery - Anticancer Agents for Colorectal Cancer	$ 283,846
U01 CA 50771-01	Hecht, Sidney University of Virginia Mechanism-Based Discovery of Novel Antitumor Agents	$ 428,587
5 U01 CA 46088-03	Johnston, Michael R. University of Colorado Health Science Center Targeted Therapy for Lung Cancer	$ 599,465
5 U01 CA 37641-05	Mendelsohn, John Memorial Sloan Kettering Cancer Center Anti-Receptor Monoclonal Antibodies in Cancer Treatment	$ 771,877
5 U01 CA 48626-02	Murphy, John R. The University Hospital, Boston Growth Factor Receptor Targeted Toxins for Leukemia/Lymphoma	$ 581,844
5 U01 CA 37606-05	Porter, Carl W. Roswell Park Memorial Institute Inhibitors of Polyamine Biosynthesis and/or Function	$ 686,389

TABLE 3, CONTINUED

5 U01 CA 40884-06	Ross, Warren E. University of Louisville Topoisomerases as New Therapeutic Targets	$ 597,219
5 U01 CA 48405-02	Wahl, Geoffrey M. The Salk Institute Detection and Curing of Amplified Genes in Human Cancer	$ 245,661
	Total	$5,447,816

TABLE 4

NATIONAL COOPERATIVE DRUG DISCOVERY GROUPS

PLANNED AWARDS

Grant Number	Investigator/Institute/Title
U01 CA 48508	Brem, Henry Johns Hopkins Hospital Controlled Release Polymers for Brain Tumors
U01 CA 52956	Cordell, Geoffrey University of Illinois at Chicago Novel Strategies for Plant-Derived Anticancer Agents
U01 CA 52955	Crews, Philip University of California Discovery of Antitumor Marine Natural Products by Mechanism-Based Approaches
U01 CA 51880	Houston, L.L. Cetus Corporation Engineered Antibreast Cancer Single-Chain FV Immunotoxin
U01 CA 52859	Levin, Victor M.D. Anderson Cancer Center Discovery and Development of Tyrosine Kinase Inhibitors
U01 CA 51908	Lippman, Marc E. Georgetown University Growth Regulation as Targets in Breast Cancer Treatment
U01 CA 52020	McCaffrey, Ronald P. Boston University Specific Therapy for TdT-Positive Leukemia/Lymphoma
U01 CA 51992	McCormick, Frank Cetus Corporation Discovery of Drugs Inhibiting Oncogenic RAS Proteins
U01 CA 52995	Powis, Garth Mayo Foundation Cancer Drugs Active Against Signal Transduction Targets
U01 CA 51946	Reisfeld, Ralph A. Research Institute of Scripps Clinic New Tumor Models for the Development of Immunotherapy
U01 CA 51958	Steplewski, Zenon The Wistar Institute Radioisotope-Antibody Conjugates for Cancer Therapy

TABLE 4, CONTINUED

U01 CA 53001	Valeriote, Frederick Wayne State University Discovery of New Anticancer Agents from Natural Products	

ANNUAL REPORT OF THE PHARMACOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

The Pharmacology Branch (PB) is involved with two basic aspects in the preclinical drug developmental program: detailed therapeutic studies and pharmacokinetic studies, including method development, on candidate agents for cancer and AIDS treatment. Via contract mechanisms, 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 in both human xenograph and murine tumor models are currently being used. For AIDS drugs, murine retroviruses (Rauscher leukemia - R-MuLV and LP-BM5) are the current useful models. A new lentivirus, bovine immunodeficiency - like virus (BIV), is being explored this year as a potential model in Dutch Belted rabbits. It carries genomic characteristics (rev, tat, and riv) similar to HIV-1. The SCID and transgenic mouse are also being considered as potential models.

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 agents' in vivo behavior.

Numerous cancer drug candidates were evaluated under the detailed therapeutic studies contract: CPE-C NSC 375575 and Penclomedine NSC 338720 work continues, Pyrazoloacridine NSC 366140 was completed with a summary prepared for the IND application. New agents introduced and being evaluated during this period include the camptothecin analogs, ellipticinium derivatives, a cholera toxin and some cytochalasins. Several drug combination studies were also conducted involving: (1) cisplatin and aphidicolin glycinate; (2) BCNU and O^6- methylyguanine (an inhibitor of alkylguanyltransferase).

In the detailed therapeutic studies contract for AIDS, initial agents identified by the anti-HIV FCRF screen are undergoing evaluation in murine retrovirus models both in vitro and in vivo. Background data continues to be obtained for the in vivo and in vitro activity of AZT against the Rauscher MuLV and the LP-BM5. Polyoxometalate NSC 622102, Stibene aldehyde NSC 618121 and several discreet compounds NSC 625487D, 624231D, 627708D, 615985D and 619179D have been evaluated for in vivo activity against both R-MuLV and LP-BM5. Four discreet ATA polmer fractions were evaluated with R-MuLV only and Oxathiin carboxanilide NSC 615985 and NSC 616419D were evaluated with the LP-BM5 only.

During this year three AIDS pharmacology contracts and three cancer pharmacology contracts continue in their operation. Seven novel compounds with demonstrated in vitro anti-HIV activity were studied during this period on the AIDS contracts. Pharmacokinetic investigations in mice were completed for a novel nucleoside, deoxythiacytidine NSC 620753, as well as a synthetic organic compound, oxathin carboxanilide NSC 615985. Analytical methods development and pharmacokinetic studies were also completed for four discreet anti-HIV compounds NSC 618121D, 619858D, 622102D, and 624958D). An additional discreet compound NSC 629243D was recently started and work continues. On the cancer pharmacology contracts, method development and mouse pharmacokinetics were completed on O^6- methylyguanine, the anti-motility agent NSC 609974, rapamycin and its C(28)-N,N- dimethylglycinate prodrug, micronized merbarone,

penclomedine NSC 338720 and ellipticine NSC 627505 and its 9-OH derivative NSC 264137. During this period work was begun on several new agents. Camptothecin and several of its analogues of interest to DTP, 9-glycinamido-camptothecin NSC 619232, 9-aminocamptothecin NSC 603071, 10,11-methylenedioxycamptothecin NSC 606174 and hycamptamine NSC 609699 are being studied for method development and comparative pharmacokinetics. Work has been initiated to develop an Elisa method and to study in mice the pharmacokinetics of Cholera toxin in mice. In a collaborative arrangement with CTEP, because of a perceived need for rapid method development for the agent tetraplatin NSC 363812, one of our contractors has been assigned the task of devising a method for the measurement of this agent and to confirm its utility with pharmacokinetic studies in mice.

PUBLICATIONS BY STAFF

Jayaram HN, Lui MS, Plowman J, Pillwein K, Reardon MA, Elliott WL, Weber G. Oncolytic activity and mechanism of action of a novel L-cysteine derivative, L-cysteine ethyl ester, S-(N-methylcarbamate)monohydrochloride, Cancer Chemother Pharmacol 1990; (in press).

Mucci-Lorusso P, Polin L, Bissery MC, Valeriote F, Plowman J, Luk GD, Corbett TH. Activity of batracylin (NSC 320846) against solid tumors of mice, Invest New Drugs 1989;7:295-306.

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;81:1088-1092.

Zaharko DS, Kelley JA, Tomaszewski JE, Hegedus L, Hartman NR. Cyclopentenyl Cytosine: Interspecies predictions based on rodent plasma and urine kinetics, Invest New Drugs 1990;(in press).

PUBLICATIONS BY CONTRACTORS:

Ames MM, Loprinzi CL, Van Haelst-Pisani CM, Collins JM. Clinical pharmacologic evaluation of pirozantrone (Oxantrazole) in a phase I trial incorporating a pharmacologically guided dose escalation scheme, Cancer Research 1990; (in press).

Ames MM, Mathiesen DA. High-Performance liquid chromatographic assay, Preclinical pharmacologic studies with the experimental antitumor agent batracylin, J Chromatog Biomed Appl 1989;491:488-494.

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;24:277-283.

Chapman DE, Moore DJ, Melder DC, Breau A, Powis G. Isolation identification and biological activity of a phyllanthoside metabolite produced in vitro by mouse plasma, Cancer Chemother Pharmacol 1989;25:184-187.

El Dareer SM, Tillery KF, Kalin JR, Hill DL. Disposition of 2',3'-dideoxyadenosine and 2',3'-dideoxyinosine in mice, Invest New Drugs 1989; (in press).

El Dareer SM, Tillery KF, Rose LM, Struck RF, Hill DL. Disposition and metabolism of carbovir in mice dosed intravenously or orally, Drug Metabolism and Disposition 1990; (in press).

Frank SK, Mathiesen DA, Szurszewski M, Kuffel MJ, Ames MM. Preclinical Pharmacology of the anthrapyrazole analog oxantrazole (NSC-349174), Cancer Chemother Pharmacol 1989;23:213-218.

Harrison SD, Plowman J, Dykes DJ, Waud WR, Griswold DP Jr. Schedule dependence activity against natural metastases and cross-resistance of pyrazine diazohydroxide (sodium salt, NSC 361456) in preclinical models in vivo, Cancer Chemother Pharmacol 1990;25:425-429.

ANNUAL REPORT OF THE TOXICOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

The objectives of the Developmental Therapeutics Program center on the discovery and preclinical development of agents with anticancer, and 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 *in vitro* 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 four new antineoplastic drugs or analogs and eight anti-HIV agents were in the IIA phase of preclinical toxicology. Two of these drugs were placed on hold pending results from ongoing clinical trials of analogs and further studies on a third drug (carbovir) were dropped due to licensing of the drug to Glaxo. Two of the other drugs moved from IIA to IIB and the studies on one of these (DTHC) were concluded prior to completing the INDA-directed studies due to licensing of this drug to Glaxo.

Of the three cancer drugs currently at the IIB level, one of these (cyclodisone) was dropped due to toxicity and formulation problems. Two other drugs in Stage IIB have completed evaluation and are now in Stage III. These were pyrazoloacridine and cyclopentenyl cytosine.

In addition to studies on these drugs in preclinical development, studies on three other drugs already in clinical trials were requested by CTEP and the Pediatric Branch, COP, and have been initiated. These involve combination studies with MGBG, tegretol and possible DFMO to define the conditions for

hepatotoxicity seen in the clinic; a combination study with carboplatin and BSO; and an oral study with Ara AC.

DRUGS IN DN STAGE IIA ON WHICH PRELIMINARY TOXICITY STUDIES WERE PERFORMED

ANTICANCER

MORPHOLINODOXORUBICIN	354646[a]
CYANOMORPHOLINODOXORUBICIN	357704[a]
9-AMINOCAMPTOTHECIN	603071
MERCK L651582	609974D

ANTI-HIV

CARBOVIR	614846[b]
OXATHIIN CARBOXANILIDE	615985
DISCREET	618121D
DISCREET	619179D
DISCREET	619858D
2′-DEOXY-3′-THIACYTIDINE	620753[b,c]
TERBIUM POLYOXOMETALATE	622102D
DISCREET	629243D[c]

DRUGS IN DN STAGE IIB ON WHICH FULL-SCALE TOXICITY STUDIES WERE PERFORMED

ANTICANCER

PENCLOMEDINE	338720
CYCLODISONE	348948[d]
8-CHLORO CYCLIC AMP	614491

DRUGS THAT COMPLETED EVALUATION IN DN STAGE IIB AND MOVED INTO STAGE III

ANTICANCER

PYRAZOLOACRIDINE	366140
CYCLOPENTENYL CYTOSINE	375575

DRUGS IN DN STAGE III OR BEYOND ON WHICH TOXICITY STUDIES WERE PERFORMED

ANTICANCER

MGBG/TEGRETOL COMBINATION	032946
CARBOPLATIN/BSO COMBINATION	241240
ARA AC	281272

[a] PLACED ON HOLD.
[b] STUDIES WERE CONCLUDED DUE TO LICENSING OF DRUG.
[c] MOVED TO STAGE IIB.
[d] DROPPED.

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 1990, the Branch continued an innovative project designed to provide more definitive information on human toxicity of investigational agents and to provide additional information on the antiviral activity of potential new drugs to treat AIDS. This project compares the 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 rodent, canine and human lymphocytes and monocytes, both freshly isolated and in immortalized cell lines. The other aspect of this project is attempting to determine antiviral activity of serum samples taken from animals dosed with promising new AIDS therapies. These studies are still in the development stage.

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.

<u>Publications</u>:

Manuscripts:

Du DL, Volpe DA, Grieshaber CK, Murphy MJ Jr. L-Phenylalanine mustard (L-PAM) and L-Buthionine sulfoximine (L-BSO): Effects on murine and human hematopoietic progenitor cells in vitro. Cancer Res 1990. (In press)

Du D, Volpe D, Grieshaber CK, Murphy MJ Jr. In vitro myelotoxicity of 2′,3′-dideoxynucleosides on human hematopoietic progenitor cells. Exp Hematol 1990. (In press)

McGowan JJ, Tomaszewski JE, Cradock J, Hoth D, Grieshaber CK, Broder S, Mitsuya H. An overview of the preclinical development of an antiretroviral drug, 2′,3′-dideoxyinosine (ddi). Review of Infectious Diseases 1990. (In press)

Collins JM, Grieshaber CK, Chabner BA. Pharmacologically-guided phase I clinical trials based upon complementary preclinical development. J National Cancer Inst 1990. (In press)

Zaharko DS, Kelley JA, Tomaszewski JE, Hegedus L, Hartman N. Cyclopentenyl Cytosine: Interspecies predictions based on rodent plasma and urine kinetics. Invest New Drugs (IND) 1990. (In press)

Book Chapter:

Grieshaber CK. Prediction of human toxicity from animal studies. In: G Powis, M Hacker, eds. Mechanisms of toxicity of anticancer drugs: a study in human toxicity. New York: Pergamon Press, 1990. (In press)

ANNUAL REPORT OF THE PHARMACEUTICAL RESOURCES BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1989 to September 30, 1990

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 27 contracts with a combined annual budget of approximately ten 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 seven senior professionals, one procurement agent and two secretarial personnel. The classification of the senior professionals is as follows: four 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 thirteen 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 resource 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 five analytical contracts representing a combined 22 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 rodent models, 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 three 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 five 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. 88-2141) which is updated periodically.

Goals and Accomplishments

During the past year the Branch has been actively involved in the development of emulsion and microdispersions for intravenous administration.

The branch continued to be 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 new agents from the AIDS and cancer screens will 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 continue to concentrate on evaluating new parenteral drug delivery systems such as with liposomes, emulsions and micro particles for intravenous use.

Publications by Staff

1. Flora KP, Software review: MINSQ 2.3, Am J Hosp Pharm 1989;46:1052-54

2. Toledo MM, Cadwallader DE, Trissel LA and Flora KP: Stability of pibenzimol HCl in commonly used infusion solutions and after filtration, Am J Hosp Pharm 1989;46:2043-46

3. Duafala ME, Kleinberg ML, Nacov C, Flora KP, Hines J, Davis K, McDaniel A and Scott D: Stability of morhphine sulfate in infusion devices and containers for parenteral administration Am J Hosp Pharm 1990;47:143-6

4. Duafala ME, Kleinberg ML, Nacov C, Flora KP, Hines J, Davis K, McDaniel A and Scott D.: Stability of heroin hydrochloride infusion devices and containers for parenteral administration Am J Hosp Pharm 1990;47:377-81

5. Flora KP, The discovery and preclinical development of anticancer and anti-AIDS drugs at the national cancer institute, Cancer Bull, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07183-04 PRB

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 cancer and the AIDS virus are being systematically investigated to obtain structural and electronic properties which may help to elucidate the mechanism of their action and thus lead to improved analogs. The x-ray structures of 2',3'-dideoxyinosine (DDI) and Carbovir are being determined. Strain energies and quantum calculations are being carried out on these compounds and on camptothecin.

☆U.S. GOVERNMENT PRINTING OFFICE: 1991-288-231