Intramural Activities Volume I

October 1, 1990- September 31, 1991

91 annual repor

Division Of

Cancer Treatment

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

NATIONAL CANCER INSTITUTE

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ANNUAL REPORT

October 1, 1990 through September 30, 1991

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ANNUAL REPORT

DIVISION OF CANCER TREATMENT

October 1, 1990 through September 30, 1991

The responsibility of the Division of Cancer Treatment (DCT) of the National Cancer Institute (NCI) is identification, development and evaluation of new therapies aimed at the control and cure of cancer and, increasingly over the past several years, the acquired immunodeficiency syndrome (AIDS). In addition to the Office of the Director (OD), the Division has five major components: the Developmental Therapeutics Program (DTP), the Cancer Therapy Evaluation Program (CTEP), the Radiation Research Program (RRP), the Clinical Oncology Program (COP), and the Biologic Response Modifiers Program (BRMP). Research supported by the DCT is conducted in intramural laboratories and in the clinics and in-patient facilities of the NIH Clinical Center and in extramural academic centers, hospitals and private businesses throughout the United States and in other countries. Extramural projects are supported by grants, contracts, and cooperative agreements. The treatment-oriented emphasis in research efforts of the DCT encompass a spectrum of activities, including gene therapy, immunotherapy and biologic response modifiers, as well as the traditional disciplines of chemotherapy, surgery and radiation. Active interchange of ideas and continuous cross-fertilizing collaborations between clinicians and laboratory scientists are key to the advancement of knowledge and improvement of therapeutic outcome; in fact, many DCT-supported laboratory scientists in both the intramural program and the extramural research community are also active clinicians with integrated laboratory and clinical efforts. An orderly, stepwise development process of new therapeutically active agents or new treatment concepts is followed. A major effort of the Division is the identification of new active agents. When a compound or extract, usually a natural product obtained from plant, marine or microorganism sources, is found to have antitumor activity or anti-HIV activity, a series of preclinical development steps ensue, including purification and chemical characterization, formulation for delivery to animals, and safety testing in animal models. New agents that are found to have acceptable toxicity profiles in animals are then submitted to the Food and Drug Administration (FDA) for approval for beginning human trials, which begin with Phase I (toxicity) testing, proceed to Phase II (identification of specific antitumor activity) and, for agents active in Phase II, subsequent Phase III comparisons with best existing therapies, either alone or in combination with other active agents. Although the process can be slow, improved technology for preclinical evaluation and administrative fast-tracking of promising new anticancer and anti-AIDS drugs are increasingly allowing expeditious approval for human testing.

The DCT functions with the scientific advice and consent of the Board of Scientific Counselors (BSC) (Table 1), which comprises leading scientists representing the areas of basic and clinical science particularly important to the research mission of the Division, including medical, surgical and radiation oncology, immunology, hematology, molecular biology, medicinal chemistry and pharmacology, and diagnostic radiology. The Division Director and the Program Associate Directors rely on the BSC for advice on scientific, administrative and fiscal management of the Division, and BSC consent is required for new scientific initiatives.

TABLE I. DCT BOARD OF SCIENTIFIC COUNSELORS

Name	Affiliation	Term of Appointment
John E. Niederhuber, M.D. (Chairman)	Johns Hopkins University School of Medicine	1986-1991
Robert L. Baehner, M.D.	Children's Hospital, Los Angeles	1989-1992
Charles M. Balch, M.D.	M.D. Anderson Hospital	1987-1991
Paul P. Carbone, M.D.	Univ. of Wisconsin Cancer Ctr.	1989-1993
James D. Cox, M.D.	M.D. Anderson Cancer Center	1987-1991
Phillip Crews, Ph.D.	Univ. of California, Santa Cruz	1989-1993
Mark T. Groudine, M.D., Ph.D.	Fred Hutchinson Cancer Res. Ctr.	1986-1991
Robert W. Holden, M.D.	Wishard Memorial Hospital	1991-1994
William M. Hryniuk, M.D.	Ontario Cancer Foundation	1990-1992
Frank M. Huennekens, Ph.D.	Res. Institute of Scripps Clinic	1989-1991
Loretta M. Itri, M.D.	R.W. Johnson Pharmaceutical Research Institute	1991-1994
Donald W. Kufe, M.D.	Dana-Farber Cancer Institute	1991-1994
Elliott C. Lasser, M.D.	Univ. of California, San Diego	1991-1994
Ronald Levy, M.D.	Stanford University Medical Ctr.	1989-1993
Victor Ling, Ph.D.	Ontario Cancer Institute	1991-1994
David W. Martin, Jr., M.D.	DuPont Merck Pharmaceutical Co.	1991-1994
JoAnne Stubbe, Ph.D.	Massachusetts Inst. of Technology	1989-1993
Ralph R. Weichselbaum, M.D.	Univ. of Chicago Medical Center	1989-1993

The scientific accomplishments of the Division over the past year will be presented in detail within this report. Fiscal Year 1991 saw several important advances by intramural DCT scientists and by extramural scientists supported by the Division. These include the first trials in humans of gene therapy (in which "designer cells," modified by in vitro gene transfer to have enhanced ability to kill cancer cells or attract other cytotoxic cells to the tumor, are re-infused into the patient); identification and development of several new anticancer agents by the drug discovery effort; advances in understanding the role of tumor suppressor genes in lung cancer, which present potential new targets for anticancer drug development; improvements in the understanding of the role of the multidrug-resistance gene in mediating resistance to cancer chemotherapy and advances in the ability to reverse the MDR phenotype of cancer cells; continued advances in the rational use of cytokines and monoclonal antibodies as part of cancer therapy; and improvements in the treatment of both adult and pediatric AIDS. While these and other scientific accomplishments to be described below give reason for optimism, review of recent cancer statistics indicates that incidence and deaths from the major types of cancer have not improved over the past several years, underscoring the importance of redoubled efforts to search for innovative strategies for therapy and improved understanding of the enemy.

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)

- Mace Rothenberg, M.D., Special Assistant for Clinical Affairs, resigned in June to accept the position of Executive Director, Southwestern Oncology Group, San Antonio, Texas.

- Dwight Kaufman, M.D., Ph.D., was appointed Acting Deputy Director, DCT. Dr. Kaufman formerly served as a Senior Investigator in the Radiation Oncology Branch, COP.

- Ruthann Giusti, M.D. was appointed Special Assistant for Clinical Affairs. Dr. Giusti formerly served as a Medical Staff Fellow in the Medicine Branch, COP.

B. Biological Response Modifiers Program (BRMP)

- Dr. Ronald G. Steis left his position as Medical Officer, Clinical Research Branch, to continue his career in the private sector.

- Dr. Barry L. Gause, formerly with the Howard University Cancer Center, was recruited as a Medical Officer in the Clinical Research Branch.

C. Cancer Therapy Evaluation Program (CTEP)

- Dr. Susan Arbuck, formerly Associate Professor of Medicine, State University of New York at Buffalo, has been recruited as a Clinical Research Scientist in the Developmental Therapy Section, Investigational Drug Branch.

- Mr. Clarence Fortner, formerly Head, Drug Management Authorization Section, IDB, retired from the PHS to join the Adria Pharmaceutical Company.

- Mr. Alfred Fallavollita, Jr., was appointed to the position of Head, Drug Management Authorization Section, Investigational Drug Branch.

- Dr. Malcolm Smith has been appointed as a Senior Investigator in the Pediatric Section, Clinical Investigations Branch.

- Dr. Timothy Moore resigned as a Medical Officer in the Medicine Section, Clinical Investigations Branch, to join Medical Oncology Associates in Pittsburgh.

- Dr. Edward Trimble joined the Surgery Section, Clinical Investigations Branch, as a Senior Investigator; he was formerly with the Memorial Sloan-Kettering Cancer Center.

- Dr. Jan Casadei, formerly a research scientist with IGEN Inc., Rockville, was recruited as a Chemist in the Drug Regulatory Affairs Section, Regulatory Affairs Branch.

- Ms. Elizabeth Moore joined the Drug Regulatory Affairs Section, Regulatory Affairs Branch, as a Pharmacist; she was formerly with the National Inst. of Allergy and Infectious Diseases.

- Mr. Michael Montello transferred from the NIH Clinical Center Pharmacy to the Drug Management and Authorization Section, Investigational Drug Branch, as a Clinical Research Pharmacist.

D. Clinical Oncology Program (COP)

- Sir Andrew Patrick Forrest completed his Visiting Scientist appointment in the Medicine Branch and resumed his position as Professor Emeritus, Dept. of Surgery, University of Edinburgh, Scotland.

- Dr. Bach-Yen Nguyen joined the Medicine Branch as a Senior Clinical Investigator; she was formerly a Senior Clinical Associate in NIAID.

- Dr. Gary H. Weiss left the Surgery Branch to accept a position as Staff Urologist at the Long Island Jewish Medical Center, New Hyde Park, New York.

- Dr. Paul Aebersold left the Surgery Branch to accept a position as a Microbiologist at the Center for Biologic Evaluation and Research, FDA.

- Dr. Attan L. Kasid left the Surgery Branch to assume a teaching and research position.

- Dr. John D. Minna left his position as Chief, NCI-Navy Medical Oncology Branch, to accept the position as Director, Simmons Comprehensive Cancer Center, Univ. of Texas Southwestern Medical Center, Dallas.

- Dr. Adi Gazdar left the NCI-Navy Medical Oncology Branch to join the Simmons Comprehensive Cancer Center, Univ. of Texas Southwestern Medical Center, as Professor of Pathology.

- Dr. Bruce Johnson accepted appointment as the Acting Branch Chief, NCI-Navy Medical Oncology Branch.

- Dr. Carmen Allegra was appointed Branch Chief, NCI-Navy Med. Oncology Branch.

- Dr. James Mulshine left his position as Chief, Biotherapy Section, NCI-Navy Medical Oncology Branch, to become a Branch Chief with the Division of Cancer Prevention and Control, NCI.

- Dr. Michael Birrer left his position as Senior Investigator, Biotherapy Section, NCI-Navy Medical Oncology Branch, to assume a position with the Division of Cancer Prevention and Control, NCI.

- Dr. Frederick Kaye joined the NCI-Navy Medical Oncology Branch as a Senior Clinical Investigator; he was formerly an Associate Professor with USUHS.

- Dr. Daniel Ihde left his position as Chief, Clinical Investigations Section, NCI-Navy Medical Oncology Branch, to assume the position of Deputy Director, National Cancer Institute.

- Dr. Andrew Raubitschek resigned as radiation oncologist with the Radiation Therapy Section, Radiation Oncology Branch, to accept an appointment to the medical staff of the City of Hope National Medical Center, Duarte, Calif.

- Dr. Alan H. Epstein joined the Radiation Oncology Branch as a Radiation Oncologist.

E. Developmental Therapeutics Program (DTP)

- Dr. Louis Malspeis was appointed Acting Chief, Pharmaceut. Resources Branch.

- Dr. Joseph Tomaszewski was appointed Chief, Toxicology Branch.

- Dr. Edward Sausville was appointed Chief, Lab. of Biological Chemistry.

- Dr. John Bader was appointed Chief, Antiviral Evaluations Branch.

- Dr. Paul Randazzo was recruited as a Senior Staff Fellow in the Laboratory of Biological Chemistry; he was formerly with the Hospital of the Univ. of Pennsylvania.

- Dr. Michele Evans was appointed as Senior Clinical Investigator in the Laboratory of Molecular Pharmacology; she transferred from the Clinical Oncology Program, DCT.

- Dr. Michael Alley, a Pharmacologist in the Laboratory of Drug Discovery Research and Development, was transferred to the Biological Testing Branch.

- Dr. Philip Skehan, a Research Biologist with the Laboratory of Drug Discovery Research & Development, left to accept a position with Program Resources Inc.

- Dr. David Newman was recruited as a Marine Biologist with the Natural Products Branch; he was formerly with Lederle Laboratories.

- Dr. Robert Moore, a Pharmacist in the Pharmaceutical Resources Branch, died suddenly in July.

F. Radiation Research Program (RRP)

- Dr. John Antoine resigned as Associate Director of RRP to accept a position as Professor of Radiation Oncology at Loma Linda University in California.

- Dr. Eli Glatstein was appointed Acting Associate Director for RRP.

- Dr. Helen Stone joined the staff as a Cancer Expert; she was formerly an Assistant Professor with the Department of Radiation Oncology at the Univ. of California in San Francisco.

PROGRAM REVIEWS

The Division has five scientific programs that are described in detail in their respective individual sections of this Annual Report; highlights of these programs follow:

CLINICAL ONCOLOGY PROGRAM (Associate Director, Dr. Gregory Curt)

The Clinical Oncology Program (COP) is the intramural treatment research arm of the NCI. The program comprises six branches, which conduct basic and clinical research in adult and pediatric oncology and AIDS, infectious diseases in the immunocompromised host, clinical pharmacology, immunology, surgery, and radiation therapy and radiation biology. A particular strength of the COP is integration of clinical and laboratory efforts within every branch. All of the Branch Chiefs and most of the Section Leaders and Senior Investigators are both laboratory scientists and physicians, and each brings both points of view to both laboratory and clinical endeavors. The COP branches include the Medicine Branch (Chief, Dr. Robert Wittes), the NCI-Navy Medical Oncology Branch (Chief, Dr. Carmen Allegra--named at the end of the year), the Surgery Branch (Chief, Dr. Steven A. Rosenberg), the Radiation Oncology Branch (Chief, Dr. Eli Glatstein), the Clinical Pharmacology Branch (Chief, Dr. Charles E. Myers), and the Pediatric Branch (Chief, Dr. Philip Pizzo). In addition, a laboratory under the supervision of NCI Director Dr. Samuel Broder operates under the auspices of the Office of the Associate Director (OAD) and is directed at identifying new treatments for AIDS. The OAD also supports a Biostatistics Data Management Section supervised by Dr. Seth Steinberg.

Medicine Branch

Clinical activities of the Medicine Branch focus on four general areas of adult malignancy: 1) breast cancer, 2) colorectal cancer, 3) lymphoma, and 4) ovarian cancer. In addition, clinical trials in AIDS patients have been conducted by the Medicine Branch since 1982 and all of the currently available anti-retroviral therapies were first tested in patients on the Medicine Branch. The Branch participates with the NCI-Navy Medical Oncology Branch and the Biological Response Modifiers Program in an American Board of Internal Medicine-certified oncology training program that has a long history of training top scientists and academic medical oncologists. In addition to their interest in developing improved therapies for the above-listed specific diseases, clinical researchers in the Branch are interested in several areas of treatment that extend across multiple disease categories, including new drug development and Phase I testing, use of interferon and other cytokines in conjunction with chemotherapy in order to improve therapeutic efficacy, modulation or reversal of the multidrug-resistance phenotype, use of monoclonal antibodies conjugated with radioisotopes, and high-dose intensity chemotherapy trials using autologous bone marrow transplant. Laboratory research in the Branch is generally closely integrated with clinical research and includes studies of the biochemistry and cellular and molecular biology of thymidylate synthase and its interaction with 5-FU, leucovorin, and interferon; studies of methotrexate resistance, resistance to platinum compounds, and p-glycoprotein-mediated multidrug resistance; and studies of transcriptional regulation of the c-myc oncogene.

New Therapy Evaluations

Trials of taxol plus G-CSF in refractory ovarian cancer have confirmed significant response rates and the ability to double taxol dose with CSF rescue. Currently, trials of taxol in combination with cisplatin in ovarian cancer and taxol combined with doxorubicin in breast cancer are under way. Tetraplatin, pentosan, D1694 (thymidylate synthase inhibitor), piroxantrone + G-CSF, and fazarabine are being evaluated in Phase I or Phase II trials. Interleukin-1 was introduced late in the year as a bone marrow protecting agent prior to highdose ICE chemotherapy combined with ABMT. Interleukin-3 and monoclonal antibodies conjugated with ^{177}Lu or ^{131}I, against breast and colon cancer cells, will be introduced shortly.

Laboratory-Integrated Clinical Trials

1. Trials of interferon, 5-fluorouracil and leucovorin in colon cancer and of 5-FU, leucovorin and GM-CSF in various gastrointestinal malignancies with response, pharmacokinetic and toxicity evaluations integrated with biochemical and molecular biological studies of thymidylate synthase (TS) regulation are under way. Correlations of TS levels in clinical specimens with 5-FU response in collaboration with NSABP are also being sought.

2. Continuous infusion of natural-product chemotherapy agents combined with R-verapamil to reverse multidrug resistance in refractory lymphoma is demonstrating significant response rates. Correlative studies with levels of pgp-170 in tumor biopsy specimens indicate resistance reversal in P-170-positive tumors. Multidrug resistance reversal in other tumor types is planned.

3. High-dose chemotherapy in conjunction with hematopoietic growth factors (either G- or GM-CSF) or ABMT are under way in advanced breast cancer, refractory ovarian cancer, lymphoma, and gastrointestinal cancers.

Laboratory Studies

1. Studies in methotrexate-resistant cells show folate transport defects associated with alterations of folate-binding proteins. Two forms of the folate-binding protein have been cloned in the Branch, and both the genomic and cDNA structures and the regulatory characteristics are subjects of intense investigation.

2. Several studies of the mechanisms of multidrug resistance are under way, including analyses of mutations and amplifications of the mdr1 gene, studies of mdr gene regulation and specific down-regulators of mdr mRNA expression, and studies of pharmacological inhibitors of the pump.

3. Studies of platinum resistance and particularly of mechanisms of repair of platinum-DNA adducts are under way.

4. Four DNA sequence motifs within intron 1 of the c-myc oncogene have been shown to be important for transcriptional regulation. These elements bind nuclear proteins (MIF 1-4), resulting in termination of transcription. Mutations and deletions within this regulatory region have been found in Burkitt's lymphoma

cells and are thought to be associated in part with the loss of myc regulation in these malignant cells. Studies of these intronic regulatory sequences and the nuclear binding factors are under way.

5. Medicine Branch investigators have identified a unique mechanism of feedback translational regulation of TS by the enzyme, with relief from inhibition by binding of FdUMP to enzyme.

NCI-Navy Medical Oncology Branch

Clinical efforts in the NCI-Navy Medical Oncology Branch have focused largely on treatment of lung cancer and mycosis fungoides and on new drug testing. Dr. Carmen Allegra, who was named Branch Chief near the end of the calendar year, will be bringing a major interest in treatment of gastrointestinal malignancy to the Branch. Long-standing laboratory projects focusing on understanding the molecular pathobiology of lung cancer continue, including cloning of new cancer-related genes and studies of oncogenes and tumor suppressor genes. In addition to basic biological questions, several studies focus on correlations of gene structure and expression with clinical outcomes. These research activities are important not only for enhancing the understanding of carcinogenesis and factors contributing to the behavior of cancer cells, but also may provide the potential for innovative therapies. Studies are also under way to characterize the molecular events that occur in the bronchopulmonary epithelium in the premalignant state. These studies will enhance understanding of the events leading to malignant transformation and may provide tools for early detection of cancer in cancer-susceptible individuals.

Clinical Accomplishments

1. In collaboration with the Clinical Pharmacology Branch, a trial of recombinant IL-2-diphtheria toxin fusion protein in mycosis fungoides and other T-cell lymphoproliferative diseases that express the IL-2 receptor was initiated. Responses have been noted, although it is too early to quantify results.

2. Studies of immunohistochemical expression of a large number of antigens in lung cancers identified a panel of six neuroendocrine markers that correctly identify lung cancer cells in 95% of cases. Positive staining with this panel of markers may predict for response (and also for earlier metastases) in non-small cell lung cancer, although the numbers of patients tested prospectively are small. The significance of this project is that it may be possible to identify responsive subsets of patients before treatment.

3. A randomized trial of high-dose vs. standard-dose cisplatin and etoposide in extensive-stage small-cell lung cancer showed no difference in response rates but significantly less bone marrow toxicity with the standard-dose regimen, which gave response rates as good as any previous regimen used in the Branch.

4. Cell lines established from SCLC patients who had had clinical hyponatremia were found to express mRNA for atrial natriuretic factor (ANF) and/or arginine vasopressin (AVP), suggesting a second possible mechanism for paraneoplastic hyponatremia in patients with SCLC in addition to SIADH due to the previously described and well-characterized "ectopic" release of AVP, i.e., "ectopic" release of ANF.

5. Dihydrolenperone, a new agent with activity against human lung cancer cells in an in vitro assay, has been tested in a Phase I trial. Other Phase I or II trials have included 4-ipomeanol, hepsulfam, and ^{90}yttrium-labeled T101 antibody (against T-cell antigens).

Laboratory Accomplishments

1. Recent efforts to identify critical genetic events in the pathogenesis of lung cancer have focused on the role of tumor-suppressor genes, particularly p53 and Rb, and a continued effort to locate the recurrently deleted gene on chromosome 3. The retinoblastoma (Rb) gene mutants having defective phosphorylation and oncoprotein binding are being characterized. There is evidence that the normal Rb protein may bind to the L-myc protein; efforts are also under way to clone a second 200 kD cellular protein that binds to a specific region of the L-myc protein.

2. Studies of induced differentiation in an erythroleukemia cell line and F9 teratocarcinoma cell line have demonstrated that, irrespective of the differentiating agent, down-regulation of the c-myc gene is essential and that transfection of either c-myc, L-myc, or N-myc blocks inducer-mediated differentiation.

3. Studies of the mechanisms of site-specific recombination between different segments of the T-cell receptor gene demonstrate that these events are much more likely in the T cells of individuals with ataxia-telangiectasia than in normal T cells, and suggest that translocations leading to lymphoid malignancies may be related to this phenomenon. An acquired, transient "AT-like" susceptibility to gene rearrangement and, by inference, possible translocations were seen in the cells of individuals exposed to pesticides and herbicides that have been suggested to be lymphogenic.

Surgery Branch

Investigators in the Surgery Branch have been at the forefront in gene therapy trials in humans, in addition to continuing highly innovative clinical trials of adoptive immunotherapy using interleukin-2 in combination with tumor-infiltrating lymphocytes (TIL) or lymphokine-activated killer (LAK) cells. A very active laboratory-based research effort supports and guides these clinical trials. Studies of retroviral-mediated gene transfer of lymphokine genes into TIL cells and into tumor cells have led to the first four trials in humans of genetically modified cells. A major effort is under way to identify and clone tumor-specific antigens recognized by TIL cells and to characterize the determinants of tumor antigen recognition, particularly the role of MHC Class I determinants.

Clinical Accomplishments

1. Following the now-completed trial in 10 melanoma patients of TIL transduced with a bacterial marker gene in order to follow the distribution and time-course of TIL, and a trial done in collaboration with scientists from the NHLBI in which TIL transduced with the adenine deaminase gene were introduced into a patient with severe immunodeficiency disease due to adenine deaminase

deficiency, members of the Surgery Branch embarked on the first gene therapy trial of cancer using TIL transduced with the gene for tumor necrosis factor (TNF). A second gene therapy trial in cancer patients is awaiting final approval at the end of FY 1991: tumor cells from patients with metastatic melanoma transduced, using retroviral vectors, with the genes for TNF or IL-2 will be implanted in the patients, and it is hoped that the production of lymphokines by tumor cells will stimulate an immune-cell attack, not only of the reintroduced gene-modified cells but also of the untransduced tumor cells.

2. Comparative clinical trials of IL-2 alone vs. IL-2 plus LAK in melanoma and renal cell cancer patients have demonstrated a tendency toward a survival benefit in melanoma patients treated with the combination, but no difference in renal cell cancer patients. Pilot trials utilizing IL-2 plus TIL cells in 50 patients with advanced melanoma demonstrate objective responses in 38%, including those who had previously failed other regimens utilizing high-dose IL-2.

3. Other trials with lymphokines and hematopoietic growth factors are under way, including IL-4, IL-2 plus 5-FU and leucovorin for colorectal carcinoma, a Phase I trial of PEG-IL-2, and a Phase I trial of m-CSF.

4. A Phase I trial of intrapleural, intraoperative photodynamic therapy for pleural malignancies has begun. The use of endobronchial photodynamic therapy is also being investigated.

Laboratory Accomplishments

1. Characterization of genes conferring lysability to TIL-resistant tumor cells, obtained from a subtracted cDNA library prepared from a cultured melanoma line killed by TIL and a variant subpopulation resistant to TIL lysis, is now under way. It is expected that melanoma-specific antigens recognized by TIL will be obtained. Availability of these genes may allow for tumor vaccination trials.

2. Characterization of the TIL obtained from melanoma patients reveals in vitro lytic specificity for tumor cells from which they were derived, but not for other tumors or other tissues from the same patient. This lysis is both tumor-antigen-specific and MHC-restricted, as evidenced by the following experiment: transfection of MHC Class 1 determinants (the HLA-A-A2 gene) into allogeneic melanoma cells confers the ability of HLA-A2-specific TIL to lyse the transduced melanoma cells.

3. A variety of other studies are under way in both human and murine cells aimed at better understanding of the cytolytic T-lymphocyte response, including, in addition to the nature of the specificity, the role of various cytokines, especially TNF-alpha, IFN-gamma, GM-CSF, TNF, IL-2, IL-6 and IL-7.

Pediatric Branch

Investigators in the Pediatric Branch continue to conduct carefully integrated laboratory and clinical research in the areas of pediatric malignancy, infectious diseases, and pediatric AIDS. The Branch sponsors the Infectious Disease Consult Service for the Clinical Center and collaborates with investigators from the

Medicine Branch, Surgery Branch and Radiation Oncology Branch in conducting many clinical trials in infectious diseases as well as malignancy. Clinical trials conducted by the Branch have focused on children with acute lymphoblastic leukemia, high-grade lymphomas, and sarcomas. Phase I-II trials of antiretroviral therapy in pediatric patients with symptomatic HIV infections have been at the forefront in this area. Laboratory efforts have included studies of the molecular genotypes of three subtypes of pediatric ALL, of the role of the tumor-suppressor genes in ALL, of the interaction of Epstein-Barr virus nuclear antigen with the c-myc protein in Burkitt's lymphoma, and of other molecular characteristics of B-cell lymphomas. Several studies aimed at improvement in the ability to diagnose and treat invasive fungal disease in patients with cancer and AIDS are under way.

Clinical Accomplishments

1. Treatment of ALL has focused to a large extent on improving CNS prevention with avodiance of cranial irradiation. The occurrence of isolated CNS relapse in only three of 107 patients enrolled in a study of aggressive, early intensification therapy, intensive systemic maintenance, combined with CNS-specific treatment in patients with high-risk ALL attests to the success of this approach.

2. A significant effort has been devoted to development of new chemotherapeutic and differentiating agents. Particularly, drugs with CNS activity have been evaluated, including intrathecal diaziquone (AZQ), intrathecal 6-mercaptopurine, and intrathecal mafosfamide, a preactivated derivative of cyclophosphamide. Phase I trials of piritrexim, an orally administered lipid-soluble antifolate, all-trans retinoic acid, amifostine (an agent thought to be capable of protecting the bone marrow from the myelotoxicity of melphalan and other alkylating agents), and topotecan (a new antineoplastic agent that inhibits topoisomerase-I) are under way.

3. A randomized study comparing a third-generation cephalosporin (ceftazidime) to carbapenem for initial empirical antibiotic therapy in neutropenic febrile patients demonstrated comparable efficacy for both, although more therapeutic modifications and more documented second infections were noted in the group of patients treated with ceftazidime; the imipenem-treated patients had more complications, however, including intolerance to the drug and higher incidence of complicating C. difficile infection.

4. Phase I-II studies in children with HIV infection have demonstrated the efficacy and tolerable doses of AZT and of ddI. A dose-escalation trial of combination AZT and ddI is under way. A Phase I study of recombinant soluble CD4 alone and in combination with ddI was completed, which demonstrated no benefit in terms of p24 or CD4 counts in patients treated with rCD4 alone.

Laboratory Accomplishments

1. A variety of studies are aimed at defining the molecular pathogenesis of ALL and of Burkitt's lymphoma. Point mutations in the p53 gene have been found in lymphoblasts from a significant fraction of children with ALL and from a majority of cell lines established from small, non-cleaved cell lymphoma. Enhanced c-myc transcription by Epstein-Barr virus nuclear antigen (EBNA) in

small, non-cleaved cell lymphoma, in which the immunoglobulin enhancer is juxtaposed to the c-myc gene, was observed, providing a potential mechanistic explanation for the association of EBV with Burkitt's lymphoma.

2. Antisense oligonucleotides have been used to inhibit expression of the c-myc gene protein in Burkitt's lymphoma cells, resulting in inhibition of proliferation, suggesting a possible therapeutic target.

3. Constitutive expression of the cell-cycle protein $p34^{cdc2}$ has been identified in neuroblastoma cell lines. Retinoic acid treatment results in differentiation of the cells and a decreased expression of $p34^{cdc2}$, resulting in underphosphorylation of the Rb tumor-suppressor gene. Other studies of retinoic acid-induced differentiation in neuroblastoma cells have also demonstrated decreased levels of N-myc protooncogene and a concomitant increase in secreted TGF-beta.

Clinical Pharmacology Branch

This year represents the first full year for the reformulated Clinical Pharmacology Branch, which had been merged with the Medicine Branch in 1988. The major interests of the Branch have been clinical studies of suramin in patients with prostate cancer, combinations of suramin with other agents, and the development of new therapies aimed at blocking growth-factor stimulation or signal transduction pathways. Associated with the latter goal, laboratory studies have focused on defining the *in vitro* antitumor activity and mechanisms of action of receptor antagonists, nonhydrolyzable ATP analogs, cyclic AMP analogs, phenylacetate, and heparan sulfates. A semisynthetic isoflavone shown to inhibit tyrosine kinases in cell-free systems was shown to be active against lung cancer cell lines and in nude mice xenografts. Preclinical development of this compound is being expedited. Evaluation of other tyrosine kinase inhibitors, especially herbimycin A, are also under way. In collaboration with the NCI-Navy Medical Oncology Branch, a trial of recombinant IL-2-diphtheria toxin fusion protein in T-cell lymphoma patients expressing IL-2 receptors, especially mycosis fungoides patients, was begun.

Radiation Oncology Branch

Research in the Radiation Oncology Branch continues with a closely intertwined group of investigators with a broad base of expertise, including especially clinical radiation therapy, medical oncology, radiation biology and cell biology, molecular biology, biochemistry, organic chemistry, physics, engineering, and computer science. Clinical investigators in the Radiation Oncology Branch continue to collaborate with investigators in the other NCI clinical branches in a variety of multimodality therapeutic trials, with a treatment emphasis on gliomas, lymphomas, sarcomas, breast, gastric, bladder and lung carcinomas. Standard photon and electron and interstitial techniques are used. Photodynamic therapy was developed in the Branch and is being used clinically in collaboration with investigators from the Surgery Branch to treat tumors in body cavities such as the peritoneal and pleural cavities. Studies with radiation sensitizers, particularly in gliomas, head and neck cancer and sarcomas, continue. A new class of radiation and general oxidation-stress protection agents developed by scientists in the ROB is currently in preclinical development.

Clinical Accomplishments

1. A randomized study on early-stage breast cancer comparing modified radical mastectomy with definitive radiation therapy with breast preservation has been completed. Accrual was limited to women with masses of 5 cm or less with or without nodes, and included adjuvant chemotherapy for those with positive nodes. There were no differences in survival between surgery and radiation.

2. Phase I clinical studies of photodynamic therapy in patients with ovarian and gastrointestinal cancers limited to the peritoneal cavity and of intrathoracic tumors involving the pleura following definitive resection have demonstrated that this type of therapy can be delivered safely and that local control can be effective.

Laboratory Accomplishments

1. In addition to studies in support of clinical trials, a major interest of the basic scientists in the laboratory has been the study of the phenomenon of oxidative stress, including the mechanisms of oxidative damage to cells induced by radiation, various chemotherapeutic agents, cytokines (including IL-1 and TNF), hyperoxia, inflammation, and reperfusion injury, and in studies of the mechanisms whereby cells protect themselves from oxidative injury. A long-standing interest of the lab has been in defining the role of glutathione. Recently, studies of the mechanism of protection by various cellular enzymes, including glutathione peroxidase, catalase and superoxide dismutase, using sense and antisense gene transfer techniques have begun. A major effort of the lab has centered on defining the role of nitroxides, a class of stable free radicals possessing the ability to protect cells and whole animals from a variety of oxidative stresses, including radiation, hydrogen peroxide and TNF. Nitroxides may prove to be ueful subcellular probes of mechanisms of oxidative damage, and may find clinical usefulness as radiation protectors or as protectors from other oxidative damages, including reperfusion injury.

2. Since nitroxides are long-lived free radicals, they can be detected by electron paramagnetic resonance (EPR). A new initiative of the ROB is the development of EPR imaging, which in conjunction with nitroxides having long biological half-lives (e.g., attached to intravascular proteins) would have the potential of being extraordinarily fast and sensitive in comparison with MRI. Free-radical reactions in cells or in extracellular spaces could be detected with tremendous sensitivity.

3. Investigators in the Inorganic and Radioimmune Chemistry Section continue development of radioisotopes conjugated with monoclonal antibodies, especially focusing on yttrium-90 and the alpha-particle emitter Bi-212.

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

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

intramural component and the extramural grants-management component of the BRMP are located in Frederick, Maryland. Investigators at BRMP work in close collaboration with the Developmental Therapeutics Program, the Cancer Treatment Evaluation Program and the Clinical Oncology Program in a variety of efforts.

The extramural research activities sponsored by the BRMP are planned and monitored by the investigators in the Biological Resources Branch (Dr. Stephen Creekmore, Branch Chief). BRB activities include sponsorship of Phase I and II clinical trials of biological agents and overseeing of a large grants portfolio of basic and applied investigator-initiated research projects, as well as overseeing contract-supported preclinical and clinical research.

Three basic science laboratories and a clinical branch comprise the intramural research program of the BRMP. These are the Laboratory of Molecular Immunoregulation (Dr. Joost Oppenheim, Chief), Laboratory of Experimental Immunology (Dr. John Ortaldo, Chief), Laboratory of Biochemical Physiology (Dr. Hsiang-fu Kung, Chief), and Clinical Research Branch (Dr. Dan Longo, Acting Chief). The basic and clinical research efforts are highly integrated with numerous collaborations among the intramural laboratories and the Clinical Research Branch.

Extramural Program Accomplishments

The BRMP supported 107 grants totalling nearly $29 million in FY 1990, including 77 research projects (R01) and eight program projects (P01). This grants portfolio included many internationally recognized investigators in the areas of monoclonal antibody development, basic and applied immunology, molecular biology, and studies of cytokines, lymphokines, and hematopoietic growth factors. New initiatives of the Biological Resources Branch (BRB) include development of Requests for Proposals for production of chimeric human-mouse monoclonal antibodies, recompetition of the BRM repository, support of innovative clinical trials involving complex immunological manipulations, and establishment of Master Agreements for the large-scale production of clinical-grade cancer-specific targeting agents.

Intramural Program Accomplishments

Office of the Associate Director

Distinct mechanisms for negative selection of T cells by receptor-mediated apoptosis and by glucocorticoid-induced apoptosis were discovered. Death induced by T-cell receptor activation is blocked by cyclosporin A, whereas steroid-induced death is not. Conversely, steroid-induced death is blocked by RU-486, but T-cell receptor-activated death is not. Simultaneous exposure to both antigens and glucocorticoid blocks cell death. Mutual interference with these two death signals is related to specific blocking of the action of the stimulus-specific transcription factors. The role of cytokines and of thymic epithelial-cell signaling in shaping the T-cell repertoire is also being investigated.

Laboratory of Molecular Immunoregulation

This laboratory has been at the international forefront in investigating at the molecular level the intercellular and intracellular processes that regulate host-defense mechanisms. IL-1 was shown to protect mice from the effects of radiation

or chemotherapeutic agents that generate free radicals, apparently by protecting bone marrow precursors. Studies to define the mechanism of protection are under way. The regulation of hematopoiesis by cytokines is an area under intense scrutiny. The effect of potent proliferation inhibitors such as the TGF-beta family, which act at least in part by downregulating the receptors for other stimulatory factors, is being studied. Other studies focus on the signal transduction pathways after primary stimulation by hematopoietic factors, including IL-2, IL-3, GM-CSF and erythropoietin. Evidence has been obtained that, although the receptors for each of these factors is different and does not appear to possess intrinsic tyrosine kinase activity, the activity of each is mediated through a downstream tyrosine kinase, which may be common to all. A p97 protein that may be the common kinase is currently being characterized.

Laboratory of Experimental Immunology

This laboratory conducts studies on biological response modification and the application of these studies to the treatment of cancer. A variety of studies on natural killer (NK) cells is under way, including especially the characterization of tumor-specific target antigens necessary for NK recognition of tumor cells. Investigators in this laboratory made key observations leading to the identification of the p75 high-affinity IL-2 receptor beta-chain. They are currently studying the mechanism of cytolysis by NK cells, focusing on the effects of IL-2 on a major pore-forming protein (perforin).

The development of cytokine regimens capable of permitting an increase in the maximum-tolerated doses of chemotherapeutic agents has been a major emphasis of this laboratory, research that has led directly to clinical trials. Recent studies with IL-1 demonstrating a 40% increase in tolerable dose of cyclophosphamide led directly to Phase I trials of IL-1. The role of IL-7 in stimulating early B- and T-cell proliferation and activation is currently under study. This agent holds great promise as a direct antitumor agent and in stimulating the immune system in general. Scientists in this laboratory have recently demonstrated in vivo efficacy of an antibody to P-170 glycoprotein in reversing the multidrug-resistance phenotype of mdr-expressing colon cancer cells in a xenograft model. An immunotoxin based on this antibody has recently been generated, and exciting preliminary in vitro data suggest that it may be effective in specifically killing resistant tumor cells. Other investigators are pursuing methods of enhancing the killing effects of immunotoxins, such as use of interferon to increase cell-surface antigens recognized by the toxins.

Clinical Research Branch

This Branch conducts treatment protocols for a variety of human cancers, with a focus on the evaluation of biological agents and on the treatment of lymphoproliferative diseases in collaboration with clinical researchers in the Clinical Oncology Program. One of the most important findings of the past year has been an impressive response rate obtained in patients with melanoma in response to IL-2 plus the monoclonal antibody R24, which is specific for the GD3 ganglioside expressed on melanoma cells and can fix complement and mediate a human inflammatory response. The combination of IL-2 and R24 resulted in a 45% response rate, about twice the response seen with IL-2 alone. Further studies are under way to improve the understanding of this response, particularly the mechanism of an

elevation of peripheral blood interferon-gamma in responders only, and in an attempt to improve the response rate.

Extensive clinical development of IL-1 is under way in the Branch; Phase I studies have been completed and Phase II studies to evaluate its role as an antitumor agent, as an immune stimulant and as a hematopoietic growth factor are ongoing. Other studies include ongoing collaboration with the Clinical Oncology Program for the treatment of intermediate-grade non-Hodgkin's lymphomas with "short-course" (dose-intense) ProMACE-CytaBOM, indolent lymphoma, and early-stage Hodgkin's disease.

DEVELOPMENTAL THERAPEUTICS PROGRAM (Acting Associate Director, Dr. Michael Grever)

The Developmental Therapeutics Program (DTP) is an entirely preclinical component of the DCT, with both intramural laboratories and a large extramural contracts and grants effort, charged with the discovery and development of new anticancer and anti-HIV agents for introduction into clinical trials. Major accomplishments over the past year have included full implementation of a streamlined, automated drug screening program, the development of which was begun approximately five years ago; the implementation of a computerized selection process (the COMPARE program), which utilizes a "fingerprint" of antitumor activity in the panel of 60 human tumor cell lines to identify promising new candidate drugs; the continued acquisition of new natural-product extracts by contractors around the world, with a focus on tropical plants; and major activities to increase the supply of the promising new anticancer agent taxol.

Over 20,000 tests of submitted compounds, and particularly of natural-product extracts from the natural-product repository, were screened for anticancer activity and approximately 40,000 tests were completed in the anti-HIV screen. Over 500 potentially interesting agents from the screen were forwarded to the Cancer Biological Evaluation Committee; purification, chemical characterization, and further testing in xenograft models precede toxicological testing, and for those agents with acceptable toxicological profiles, Phase I human testing ensues. Top-priority new compounds identified for development over the past year include 9-aminocamptothecin, 9-methoxyellipticinium, dolastatin 10, and "Uniroyal Junior."

Laboratory of Molecular Pharmacology

The major goal of the LMP is to explore molecular and cellular targets that may be useful in developing new drugs or strategies in the treatment of cancer. Among the targets being explored are the topoisomerases, analysis of various inhibitors of the microtubule cytoskeletal system (combrestastatins, dolastatins, colchicine analogs and halichondrins), an acridine mustard compound with both DNA intercalating and cross-linking activity, and DNA repair inhibitors.

Laboratory of Medicinal Chemistry

The LMC is involved with the design, synthesis and development of both anticancer and anti-HIV agents. New agents emanating from this laboratory include cyclopentyl cytosine, which is ready for Phase I clinical testing (which will be done in the COP). Various other nucleosides have been synthesized and evaluated for antitumor and antiviral activities. Inhibitors of both tyrosine kinase and

protein kinase C, including phenazocine, polyhydroxylated cinnamamide derivatives, and a stable analog of O-phosphotyrosine, are being evaluated.

Laboratory of Biological Chemistry

The focus of the LBC will be towards molecular-targeted approach to drug discovery. Studies of post-translational protein acylation, especially protein kinase acylation with myristic acid, isoprenoids and retinoic acids are under way, with the idea that inhibition of acylation could interfere with the normal signal transduction function of certain proteins. New directions are expected with the naming of Dr. Edward Sausville as Laboratory Chief near the end of the Fiscal Year. Dr. Sausville brings expertise in molecular biology and an interest in specific disruption of intracellular signaling events to the laboratory and close clinical ties to the Clinical Oncology Program for early clinical testing of potential innovative new agents.

Laboratory of Drug Discovery Research and Development

The focus of this Laboratory is the discovery and development of high-priority compounds from the NCI screen, and development of new assays for anticancer and anti-HIV drug discovery.

Extramural Accomplishments

The nine DTP branches are primarily extramural components of the Program, managing a large variety of grants and contracts for drug and natural-products procurement and synthesis, storage and disbursement, purification, formulation and evaluation.

RADIATION RESEARCH PROGRAM (Acting Associate Director, Dr. Eli Glatstein)

The Radiation Research Program is an entirely extramural component of the DCT whose mission is the planning, stimulation, development, administration and evaluation of basic science and clinical research in the areas of ionizing radiation and related energy forms for therapy of cancer and of various diagnostic imaging research programs. The two RRP branches--the Radiotherapy Development Branch and the Diagnostic Imaging Branch--manage large portfolios of investigator-initiated grants, cooperative agreements, program projects and contracts; RRP staff stimulate new proposals by the development of Program Announcements (PAs) and Requests for Applications (RFAs). Major initiatives over the past year: (1) two large planning grants for development of proton-beam therapy, submitted in response to an RFA, were funded; (2) seven institutions were awarded grants for development of optimal imaging strategies for diagnosis, staging and monitoring head and neck and musculoskeletal tumors (RDOG III); (3) three applications responding to an RFA for clinical diagnostic studies and tumor metabolism of brain tumors using PET and other imaging modalities were funded; and (4) three grants responding to an RFA for support of digital chest radiography in order to enhance early detection and characterization of solitary lung lesion were funded.

Major areas of grant-supported research include: (1) intraoperative radiation therapy; (2) development of radiation sensitizers and protectors; (3) hyperthermia; (4) photodynamic therapy; and (5) radioisotope-conjugated monoclonal antibodies for diagnosis and treatment.

CANCER THERAPY EVALUATION PROGRAM (Associate Director, Dr. 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 the 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 investigator-initiated grants related to cancer treatment. CTEP supported 153 grants in FY 1991 totaling over $62 million. Sixty percent of these grants were R01s and P01s totaling almost $53 million in the clinical oncology area. Four branches within CTEP include the Investigational Drug Branch (Chief, Dr. Michael Hawkins), which is responsible for sponsoring trials of investigational drugs and for evaluating them for efficacy and toxicity; the Clinical Investigations Branch (Chief, Dr. Richard Ungerleider), which is responsible for development and implementation of disease-oriented treatment strategies across the spectrum of human malignancies; the Regulatory Affairs Branch (Chief, Dr. Dale Shoemaker), which monitors the conduct of clinical trials and assures that experimental agents are used in compliance with federal regulations; and the Biometric Research Branch (Chief, Dr. Richard Simon), which provides statistical consultation to other CTEP branches, to the extramural and some intramural activities of other DCT programs, and to the statistical centers of the clinical cooperative groups.

CTEP investigators encourage the submission of clinical grant proposals through Program Announcements and Requests for Applications. Four RFAs were issued or funded in FY 1991:

1. Small Grants to Stimulate Correlative Laboratory Studies and Innovative Clinical Trials: A total of 23 R03 grant awards were made.

2. AIDS-Lymphoma Network: This RFA, supported by two other NCI divisions and the NIAID, solicited applications that would bring laboratory research efforts to the clinic to attack the problem of managing AIDS-lymphoma patients. The Network will be composed of those institutions awarded funding. Twelve grants were funded.

3. Clinical Treatment and Correlates of Upper GI Carcinoma: This RFA encourages applicants to address their research efforts toward development of new clinical therapies of upper GI carcinomas. Twenty-two applications were found to be responsive to the RFA and are scheduled for review.

4. New Therapeutic Approaches to the Treatment of Prostate Cancer: Investigators are encouraged to utilize recent laboratory advances in understanding the mechanisms of tumor growth and hormonal control to develop clinical studies aimed at improving treatment results. Applications will be reviewed in FY 1992.

A Program Announcement entitled "Cancer Therapy Research in Lung Cancer," intended to encourage clinical therapeutic studies of lung cancer, was issued.

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Definitions of Contract Groupings
FY 1991 Annual Report

Drug Development

Compound Discovery	-	Preclinical contract program focusing on the discovery and screening of potential anticancer agents. Includes both natural products and synthetics, as well as testing (screening) *in vitro* and *in vivo*.
Compound Development	-	Includes data management, pharmacology and pharmacokinetics testing, formulation and analytical profiles, and toxicological protocols to meet FDA requirements.
Clinical Support	-	Preclinical contracts 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.

Small Business Innovative Research Awards (SBIR)

(Phase I and II awards) - Contracts to support projects limited in time and amount, to develop commercial products or services.

TABLE I

DCT Contract Program for FY 1991
(Dollars in Thousands)

		TOTALS
I.	Drug Development	$45,535
	A. Compound Discovery - Subtotal	29,096
	1. Acquisition	11,035
	a. Natural Products	6,696
	b. Synthesis	4,339
	2. Screening	18,061
	a. In Vivo	7,024
	b. In Vitro	4,757
	c. Screening Support	6,280
	B. Compound Development	7,134
	1. Cancer	3,964
	2. AIDS	3,170
	C. Clinical Support	9,305
II.	Biologics Development	1,539
III.	Diagnostic Imaging	179
IV.	Radiation Development	2,616
V.	Clinical Trials - Subtotal	11,529
	A. Drug Evaluation (Phase I/II/III)	7,649
	B. Biological Evaluation (Phase I/II)	2,789
	C. Radiotherapy	653
	D. Other	438
VI.	Support to Intramural	3,759
VII.	Drug Purchases	3,700
	A. Cancer Drugs	3,258
	B. AIDS Drugs	442
VIII.	SBIR	1,044
	TOTAL DCT CONTRACTS	$69,901

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 Japanese Foundation for Clinical Research and the Japan Society for the Promotion of Science.

RICARDO INTERNATIONAL, INC. (NO1-CMO-7314)

This contract provides technical support services to the Office of the Director and other program areas of DCT in the performance of its planning, analytical tasks, and general logistical support in the development of related or otherwise required documentation and conference support activities. This contract was newly awarded in July of 1990 for a five year period.

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

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

The purpose of this contract is to produce large-scale quantities of clinical-grade monoclonal antibodies. The Contractor is expected to produce, upon receipt of a given murine hybridoma or murine cell line producing a mouse/human chimeric antibody, 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 regulations 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 BRMP-sponsored clinical investigators for the purpose of aiding in the filing of an investigational new drug is an important responsibility of the Contractor. This contract is scheduled to expire August 31, 1991.

ABBOTT 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.

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. The objectives of this study are: (1) to determine whether different dose levels of interleukin-2 (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-gamma; (3) to assess the effects of IL-2 on the development of human antimouse antibody and anti-idiotype responses; and (4) to record toxicity and antitumor effects of the regimen. The clinical trial has determined an maximum tolerated dose of 14.G2a as 20 mg IV on days 1, 3, 5, and 8. One partial response has been seen. However, significant neurotoxicity seen at doses above the maximum tolerated dose has led to a suspension of all 14.G2a trials by the Food and Drug Administration, pending further data analysis already obtained from this and other Phase I studies. Additional costs, if any, from the clinical trial are allowable under the workscope of N01-CM-97611 (Task A) and will be covered by that contract. This contract expired on March 31, 1991.

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. The clinical trial has been completed; no significant differences in immunomodulatory or antitumor effects between the intravenous and intralymphatic routes. A manuscript is being prepared. The contract expired on March 31, 1991.

Another segment of this contract is a Phase Ia/Ib study of granulocyte-macrophage 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. Positron emission tomography scanning will be performed on selected patients as a further means of assessing effects of the treatment at tumor sites. This contract is scheduled to expire on December 31, 1991.

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. After initial delays in protocol development, three protocols are planned to begin patient accrual in CY 1991: a Phase Ib trial of R24 monoclonal antibody with either granulocyte macrophage-colony stimulating factor or macrophage-colony stimulating factor; a Phase Ib trial of tumor infiltrating lymphocytes (TIL) plus IL-2 in renal cell carcinoma patients pretreated with cytokines prior to TIL harvest; and a Phase Ia/Ib trial of a monoclonal antibody which recognizes the Her/2-neu gene product. This contract was awarded September 30, 1988 and is scheduled to expire on September 29, 1993.

CLEVELAND CLINIC FOUNDATION (N01-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 that 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 may also influence the design of other protocols involving combinations of several such antimelanoma antibodies. This is a cost reimbursement type contract that was awarded June 30, 1987 and expired on June 30, 1991.

Another segment of this contract is a Phase Ib study of macrophage activation by granulocyte-macrophage colony stimulating factor (GM-CSF) in patients with lung cancer. The objectives of this study are: (1) to evaluate the activation of alveolar and tumor-associated macrophages, in comparison with

those in the peripheral blood or other tissues. Patients will undergo serial pulmonary lavage and tumor biopsy for assessment of macrophage activation and cytotoxicity; (2) to determine clinical toxicities and record antitumor responses; and (3) to study hematopoietic maturation in the blood and bone marrow, GM-CSF pharmacokinetics, induction of anti-GM-CSF antibody, and changes in cytotoxic T lymphocytes and natural killer activity. The clinical protocol has been completed. The maximal tolerated dose of GM-CSF was 250 $mg/m^2/d$ IV D1-14. Monocyte cytotoxicity increased with increasing doses of GM-CSF. No affect on pulmonary macrophage cytotoxicity was noted, however. Both monocytes and pulmonary macrophages exhibited increased cytokine gene expression (interleukin-1, interleukin-6, tumor necrosis factor-alpha) with GM-CSF therapy. No antitumor responses were seen. This contract expired on January 31, 1991.

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. Active or planned clinical protocols include a Phase Ia/Ib trial of macrophage-colony stimulating factor; a Phase Ia/Ib trial of interleukin-2 plus interleukin-4; a Phase Ib trial of the immunomodulatory effects of interleukin-3; and a Phase Ia/Ib trial of interleukin-6. This contract was awarded in the third quarter of FY 1989 and is scheduled to run to FY 1994.

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

This contract supports a preclinical and Phase Ib study of interleukin-4 (IL-4), interleukin-2 (IL-2) and anti-CD3 monoclonal antibody for cytolytic effector cell generation. The objectives of this study are: (1) to optimize conditions for *ex vivo* augmentation of effector cell activity with IL-2/IL-4/monoclonal antibody in patients receiving IL-2 in an IL-2/lymphokine-activated killer trial; and (2) to perform a Phase Ib clinical trial of IL-2/IL-4/monoclonal antibody plus effector cells using that methodology. In the clinical trial, most patients suffered disease progression while awaiting *ex vivo* expansion of tumor infiltrating lymphocytes (TIL). IL-4 appeared to inhibit TIL expansion. Of 7 patients who received TIL, 1 had a partial response, while an additional partial response was seen in another 16 patients who received IL-2 alone. The clinical trial is now being concluded. The contract was scheduled to expire March 31, 1991. Prior to this, the contractor requested, and the Project Officer approved an additional three-month extension for completion of data analysis.

HAZLETON LABORATORIES, INC. (N01-CM1-7341)

The purpose of this contract is to prepare monoclonal antibody chelates which can bind ("chelate") radionuclides. Monoclonal antibodies selected by the NCI will be conjugated with ligands that have been shown in peer-reviewed journals to be useful in chelating radionuclides for diagnostic and therapeutic purposes. Such immunoconjugates should be stable and retain their immunoreactivity. The Offeror will prepare milligram quantities (approximately 100 mg to 1g) each of purified ligand conjugated monoclonal antibodies, antibody fragments, or other targeting proteins or peptides as specified by the NCI under conditions of Good Laboratory Practice and/or Good Manufacturing Practice. Ligand conjugated monoclonal antibodies will be distributed to intramural and extramural scientists and to other scientists at the discretion of the Project Officer. It is anticipated that this contract will be awarded by July, 1991.

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 drug applications for biologics. Currently, the Contractor provides for storage and distribution of approximately 100 different biologics. The Contractor manages a repository of 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 Manufacturing 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 on September 9, 1992.

JEFFERSON MEDICAL COLLEGE (N01-CM6-7902)

Initially a Phase Ib clinical study was planned to involve 16 patients with advanced sarcomas that are refractory to standard therapy. The objectives of the study were to assess the toxicity, therapeutic efficacy, and modulation of immune parameters with tumor-specific cytotoxic T-lymphocytes ("educated" LAK cells). However, the clinical trial failed to accrue patients, and at the direction of the Project Officer, a new protocol was proposed by the Contractor. The new study will

test the immunomodulatory effects, particularily on delayed-type hypersensitivity, of monoclonal antibody R24 in patients with metastatic melanoma. The results will be used to design a trial of R24 plus a melanoma vaccine, developed by the Contractor, consisting of autologous, irradiated, haptenated cells. This contract is scheduled to expire on February 28, 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 scheduled to expire on September 29, 1993.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NOI-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 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, that 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 DCT's Biological Resources Branch of the BRMP and the Cancer Therapy Evaluation Program. This contract was awarded September 30, 1988 and is scheduled to expire on September 29, 1993.

MEMORIAL SLOAN-KETTERING CANCER CENTER (N01-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-dependent 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 $F(ab')_2$ production currently underway. This contract was scheduled to expire on June 30, 1991; because of continued delays in production of the R24 $F(ab')_2$, a further extension in the period of performance will be required.

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 mediated by the antibody. The trial will also determine the human antimouse antibody response 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 is a cost reimbursement type contract which was awarded September 30, 1987 and is scheduled to expire on December 31, 1991.

NEW YORK UNIVERSITY (N01-CM6-7895)

The initial Phase Ia/Ib clinical trial assessed effects on macrophage activation and cytotoxicity of various dose levels of granulocyte-macrophage colony stimulating factor (GM-CSF). The objectives of this study are to determine clinical toxicities, antitumor response, hematopoietic maturation in the blood and bone marrow, GM-CSF pharmacokinetics, and induction of anti-GM-CSF antibody. The Phase Ia/Ib protocol determined that up to 250 $mg/m^2/D$ of GM-CSF could be given safely for 14 days. Macrophage cytotoxicity appeared to increase with increasing GM-CSF doses. Based on these results, a Phase Ib trial of GM-CSF plus monoclonal antibody was developed and is now accruing patients. This contract was scheduled to expire on May 31, 1991, but a further extension is possible to allow the current clinical trial to continue.

OHIO STATE UNIVERSITY (N01-CM4-7666)

This Phase Ib clinical trial in metastatic renal cell cancer was designed to explore an alternative methodology using phenylalanine methyl ester for ex vivo production of lymphokine-activated killer (LAK) cells from patients being treated with interleukin-2 (IL-2) ("expanded LAK"). In the trial design, IL-2 (Cetus) is given 3 MU/m^2 by C.I. D 1-5. Leukapheresis is performed D 8-11. LAK cells are cultured and expanded for 10 days. LAK cells and IL-2 (3 MU/m^2) are given at D 18-21. The objectives of the trial are: (1) to determine toxicity and record antitumor effects; (2) to correlate LAK activity against tumor, LAK cell phenotype, and disease histology with response; and (3) to perform imaging studies with ^{111}In-labeled LAK cells in selected patients. The clinical trial has been completed. ELAK cells appeared to produce the same toxicities, cell yields, LAK phenotypes, and LAK cytotoxicity in vitro as have been seen with other LAK cell generation methods. No antitumor responses were seen, and no patient's tumor was imaged with radiolabeled cells. A subsequent trial of the effects of IL-1 plus IL-2 on cellular immune parameters is being developed by the contractor. This contract was scheduled to expire on May 31, 1991.

PITTSBURGH, UNIVERSITY OF (N01-CM6-7893

This is a Phase Ib study to determine the optimal therapeutic and immunomodulatory dose of monoclonal antibody R24 in patients with malignant melanoma. Previous studies have not determined the maximum tolerated dose or optimal dose of the antibody, though they have shown promising therapeutic results clinically. The objectives of the trial design include: (1) to determine pharmacokinetics of the R24 antibody; (2) to perform in situ antibody localization studies; (3) to assay antibody-dependent cellular cytotoxicity function of peripheral blood mononuclear cells, serum Ig levels, complement levels, natural killer activity peripheral blood leukocyte phenotype, complement-dependent cytotoxicity, phenotypic analysis of tumor infiltrating lymphocyte, binding of monoclonal antibody to Leu-11+ circulating cells, and inflammatory response mediators at the tumor site; and (4) to evaluate antitumor responses. Patient accrual is underway. This contract is scheduled to expire on November 29, 1991.

Another segment of the contract is a Phase Ib trial in melanoma or renal cell cancer, in order to evaluate a new method developed by this group for selecting adherent LAK ("A-LAK") from cultures of human peripheral blood mononuclear cells. Isolated cells are enriched for CD3-/Leu 19+ cells which have higher cytolytic activity per cell than is achieved with unselected LAK cell preparations. The clinical usefulness of this approach was tested using 3 groups of 9 patients with infusions of different dose levels of A-LAK cells, standardized by activity against unfractionated cultures of LAK cells. A-LAK is harvested from

peripheral blood, grown over 10-15 days, and reinfused on D1. IL-2 (Cetus) is given at 10^6 U/m^2/day x 5 by continuous infusion. The objectives of the trial design are: (1) evaluation of toxicities and antitumor responses; (2) ^{111}Indium-localization studies in selected patients; and (3) determination of immunological parameters during therapy. Of 160 patients screened, 47 were able to generate A-LAK cells with sufficient proliferation to allow entry into the trial. Fifteen of these patients were treated; of these, one had a partial response, and another had a partial response rendered into a complete response after surgical resection of a residual tumor mass. Treatment side effects were similar to those seen with other LAK generation methods, and include fever, hypotension, chills, mild nausea, weight gain, and tumor-associated pain. Patient accrual has been completed. This contract is scheduled to expire on January 29, 1992.

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

This contract supports Phase Ib clinical studies of monoclonal antibody, alone and in combination, with interleukin-2 (IL-2). Objectives of the trial are determination of monoclonal antibody binding, antigen modulation, pharmacokinetics, immunomodulation, and antitumor effects of the antibody and IL-2 combination. After an initially approved protocol of monoclonal antibody Lym-1 plus IL-2 for the treatment of B-cell lymphoma failed to accrue patients, the Contractor developed a protocol to study monoclonal antibody R24 plus IL-2 in malignant melanoma. This protocol is nearing final approval. This contract is scheduled to expire on March 31, 1992.

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 two (2) specific task orders under this Master Agreement will be awarded this fiscal year. One of the Master Agreement orders requires the synthesis of seventeen (17) unmodified oligodeoxynucleotides including eight (8) specific 20/21-mers, seven (7) 15-mers, a 13-mer, and a 10-mer to study the inhibition of expression of Epstein-Barr virus proteins and c-myc in Burkitts tumors. The second Master Agreement Order involves the production of 100 mg each of unsubstituted (phosphodiester) and phosphorothioate sense and

anti-sense 15-mer oligodeoxynucleotides to examine the contribution of interleukin-6 on the growth of myeloma cells and neuroepithelioma cells in vitro and in vivo.

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 five-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 antimouse 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 is provided by the NCI. 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. The Phase I trial determined an maximum tolerated dose of 20 $mg/m^2/d$ by five-day continuous infusion. Antitumor responses in melanoma, neuroblastoma, and osteosarcoma were observed. However, because of toxicity observed on another BRMP contract trial (N01-CM4-7679-01), all trials with 14.G2a have been temporarily suspended by the Food and Drug Administration. This is a cost-reimbursement type contract that was awarded on September 30, 1987 and was scheduled to expire on July 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 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 Division of Cancer Treatment, Biological Resources Branch, and the Cancer Therapy Evaluation Branch. This contract was awarded in May, 1989 and is scheduled to expire on May 19, 1994.

WISCONSIN, UNIVERSITY OF (N01-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 have been 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. This study has been completed. The revised regimen was less toxic than the initial, 4-repetitive-cycle regimen, but produced similar immunomodulatory effects. Objective antitumor responses were seen in about 10% of patients on this trial. 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. This is a cost reimbursement type contract which was awarded on September 30, 1987, and expired on January 29, 1991.

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. Three cohorts of patients were 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 optimal biologic dose 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-γ pharmacokinetics antigen shedding and induction of anti-IFN-γ, anti-mouse Ig, and anti-idiotype responses. The IFN-γ antigen modulation trial

demonstrated a positive effect of IFN-γ on antigen shedding. A second clinical trial to evaluate murine monoclonal antibody plus IFN-γ given systemically to patients with solid tumor, is being designed based on this experience. This trial will be conducted under BRMP contract N01-CM8-7290. This contract expired on January 29, 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. Active or planned clinical trials focus on cytokine combinations, cytokines used with monoclonal antibodies, and the use of interferons to modulate expression of tumor-associated antigens. This contract is scheduled to expire on September 29, 1993.

CLINICAL ONCOLOGY PROGRAM

GEORGETOWN UNIVERSITY (NO1-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. This contract will be recompleted in 1992.

LABORATORY ANIMAL SERVICES, INC. (N01-CM9-7596)

This four year 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 work space for the performance of experimental procedures.

MEDICAL ILLNESS COUNSELING CENTER (N01-CM8-7263)

This contract provides the Clinical Oncology Program with neuropsychological testing for children and adults with HIV infection and selected oncology control groups. The purpose is to monitor, characterize, and investigate the natural history of HIV-encephalopathy (=AIDS Dementia Complex) and to document interval changes associated with therapeutic (anti-retroviral) interventions for HIV disease. This contract is undergoing recompetition.

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

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

OTC/BIOTECHNOLOGY RESEARCH INSTITUTE (N01-CM1-0000)

This contract supports the Surgery Branch by providing tumor infiltrating lymphocytes (TIL) and human lymphokine activated killer cells (LAK) for therapeutic trials. This research is directed toward the development of adoptive immunotherapies for the treatment of cancer. This contract is currently being recompeted for a three year period.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (USUHS) (Y01-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) (Y01-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 is necessary to the research mission of the NCI-NH-BETH medical oncology program and to the undergraduate education mission of USUHS.

APPLIED GENETICS, INC. (N43-CM0-7360) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract is to develop an *in vitro* test to assess the acquisition of drug resistance by human tumors. applied Genetics proposes to test the hypothesis that human chloroethylnitrosourea (CENU) resistance can be correlated with levels of the DNA repair enzyme O-6 methyl guanine-DNA methyl transferase (MGMT) in tumors. Assaying this enzyme before and after CENU treatment may be useful in chemotherapy planning. During the Phase I period, antibodies reactive to MGMT were developed and tested in immunohistochemical assays on fixed specimens and in ELISA assays using tissue extracts. The ELISA assay showed promise for detecting MGMT at extremely low levels and may be useful in clinical settings. The immunohistochemical assay needs further development. The contractor is planning to submit a Phase II proposal to continue this endeavor.

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) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

CAPITOL TECHNOLOGY INFORMATION SERVICES (NO1-CM1-7508)

This contract supports maintenance and enhancements of the computer aspects of the following CTEP information systems: The CTEP-IS, the Adverse Drug Reaction System, and the Drug Management and Authorization Section Drug Computer System (DDCS) 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 12,000 studies in the system, with 275 trials in the CTEP or PDQ review process at any one time. About 1,200 new studies are added each year with some 1,800 amendments to active studies. The ADR system tracks Adverse Drug Reactions reported on CTEP sponsored clinical trials. ADRs can be monitored by drug, by trial, by institution or by investigator. Approximately 1000 ADR reports are added each year. The Drug Management and Authorization Section Drug Computer System (DDCS) is a data base used to verify the accuracy of drug requests, to monitor drug shipment information, required of IND sponsors by the Food and Drug Administration, and to provide user friendly access to management information for the Section and for CTEP. The system includes a MicroVAX minicomputer, a DMAS LAN that is also accessible by the contractor, and a relational data base management system (ORACLE). The DDCS interfaces with the DCRT mainframe computer to access data for the Electronic Clinical Drug Request (ECDR) system, a method used for the electronic transmission of drug requests from investigators to NCI. DCRT is also utilized for specialized report printing and for mailing label generation. Currently the DDCS is also being run at DCRT with total conversion to the MicroVAX expected by the summer of 1991. In addition to monitoring investigational drug distribution, the DDCS provides support service to the program for computer related activities. The DDCS contains about 1,000 active protocol records for which drugs are provided; 6,600 active investigator records and their registration status; approximately 160 drugs and biologic agents and their dosage forms; 30,000 drug requests are processed annually; and, about 550 investigator registrations are updated each month. Numerous special queries and hard copy reports are produced which should decrease as the MicroVAX system becomes fully active. This contract was newly awarded on December 17, 1990, for a five year term.

CHICAGO, UNIVERSITY OF (NO1-CM0-7301)

This contract is one of six contracts 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.

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) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

EMMES CORPORATION (N01-CM1-7506)

The purpose of this contract is to provide direct organizational, data management, and statistical support for specific extramural clinical trials sponsored by the Cancer Therapy Evaluation Program.

This objective involves data management personnel and facilities to support extramural Group C clinical trials sponsored by the NCI, such as fludarabine phosphate in CLL, deoxycoformycin in hairy cell leukemia, VM-26 in pediatric ALL, and levamisole in colon cancer, CTEP-sponsored and coordinated intergroup activities, and CTEP-coordinated (non-cooperative group) multicenter studies.

EMMES CORPORATION (N01-CM1-7507)

The Contractor shall assist the Clinical Investigations Branch, in managing, coordinating and monitoring NCI's extramural clinical trials program from disease and/or treatment modality perspectives.

The objectives of this contract are managing a clinical trials tracking data base, retrieving scientific information from currently available data bases (e.g., cancerline, medline),

analyzing program data to assist staff in the development of future priorities and initiatives for clinical research, and contributing to the generation of scientific (cancer-related) publications.

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. This contract expired on April 27, 1991, and was recompeted.

INFORMATION MANAGEMENT SERVICES, INC. (N01-CM6-7810)

This contract supported the maintenance of the computer aspects of the following CTEP information systems: The CTEP-IS, the Adverse Drug Reaction System, and the Drug Management and Authorization Section System (DDCS) with further development of linkages between these systems and with outside databases. This contract expired on February 17, 1991. All appropriate data, hardware and software were transferred to the new contractor, Capital Technology Information Services, Inc. funded under contract N01-CM1-7508.

JOHNS HOPKINS UNIVERSITY (N01-CM0-7302)

This contract is one of six contracts 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.

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) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

MARYLAND, UNIVERSITY OF (NO1-CM0-7303)

This contract is one of six contracts to provide a resource for the conduct Phase I clinical trials 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.

MAYO FOUNDATION (N01-CM0-7304)

This contract is one of six contracts 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.

MAYO FOUNDATION (N01-CM0-7309)

This contract is one of three contracts to provide a resource for the conduct of early and high priority clinical 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; e) to determine the spectrum of antitumor activity for new agents in selected human cancers; and f) to perform trials of high priority to the National Cancer Institute's Drug Development Program.

MEMORIAL HOSPITAL FOR CANCER AND ALLIED DISEASES (NO1-CM0-7311)

This contract is one of three contracts to provide a resource for the conduct of early and high priority clinical 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; e) to determine the spectrum of antitumor activity for new agents in selected human cancers; and f) to perform trials of high priority to the National Cancer Institute's Drug Development Program.

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) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results

obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal

cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

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) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

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 has continued to provide a simplified analytical method for Suramin to each investigator with an approved LOI to conduct a clinical study with Suramin. In addition, the Contractor prepared sets of

unknown Suramin samples and shipped them to the clinical investigators in order to validate the assay of each investigator. These studies are particularly important since Suramin has demonstrated activity in prostatic cancer patients and the development of the agent has become a high priority for

the DCT. The examination of the results of Suramin determinations at the various clinical sites suggested that the reference sample of Suramin used for quantitation, and the manner in which stock solutions are prepared, may be a primary factor contributing to observed variability. Therefore, the Contractor has determined the molar absorptivity of anhydrous Suramin Sodium to facilitate spectrophotometric standardization of the primary stock solution used to prepare plasma standards. In addition, the Contractor has developed a specific and sensitive analytical assay for Pyrazine Diazohydroxide. This contract expires in FY 91 and has not been recompeted.

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. This contract expired and was replaced by a new 8A setaside contract (NO1-CM1-7503) awarded to TASCON, Inc.

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, and Regulatory Affairs Branch, including 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).

This contract is divided into two principal parts: (1) Clinical Research/FDA compliance support which includes (a) clinical research support, (b) regulatory affairs support, and (c) drug distribution support; and (2) Protocol and Information Support which includes (a) protocol review and approval tracking and coordination, (b) protocol information tracking and dissemination, and (c) protocol results and publications tracking.

TASCON, INC. (NO1-CM1-7503)

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. This new contract is a 8A setaside contract and a follow-on to contract N01-CM2-5606.

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

This contract is one of six contracts 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.

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

This Contractor is part of the NCI IL/2-LAK Working Group (ILWG). The ILWG confirmed the NCI Surgery Branch (SB) IL-2/LAK 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 patient selection factors which are poorly understood. 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. The ILWG then evaluated IL-2 plus IFN alpha in a randomized trial versus IL-2 alone at high doses which require ICU support based on promising results obtained in preliminary studies of IL-2/IFN conducted by the Surgery Branch, NCI. The trials completed accrual but were unable to demonstrate a therapeutic advantage for the combination over IL-2 alone in patients with metastatic melanoma or renal cell carcinoma. A response rate of approximately 20% in the IL-2 alone arm of the RCC randomized study confirmed the results obtained in Surgery Branch, NCI studies, and the data was submitted as part of the cetus IL-2 licensing application to the FDA. Phase II studies of high dose IL-4 in melanoma and renal cell carcinoma and a Phase I study of OKT3 in combination with IL-2 were initiated. This contract expired on June 29, 1991, and

there are no plans for recompetition or extension, although the studies currently ongoing will be completed using funding from industry sponsors.

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

This contract is one of three contracts to provide a resource for the conduct of early and high priority clinical 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; e) to determine the spectrum of antitumor activity for new agents in selected human cancers; and f) to perform trials of high priority to the National Cancer Institute's Drug Development Program.

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

The objective of this contract is to provide a Clinical Trials Monitoring Service for Phase I/II CTEP and BRMP contract studies and certain other categories of clinical research using NCI-sponsored investigational drugs. This service has four components--(1) a central data management resource for 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 investigators 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 agent trials.

WISCONSIN, UNIVERSITY OF (N01-CMO-7306

This contract is one of six contracts 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.

AEROJET STRATEGIC PROPULSION COMPANY (NO1-CM8-7273)

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

ALABAMA, UNIVERSITY OF (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.

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: (a) congeners of confirmed screening leads, to enhance activity or potency; (b) prodrugs of active leads, with structural modifications that may provide altered pharmacokinetics, altered drug transport, improved bio-availability through increased water solubility, or increased chemical stability; and (c) 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. This contract is under recompetition.

ALDRICH CHEMICAL COMPANY, INC. (NO1-CM6-7771)

This service preparative contract provides for the resynthesis of a variety of compounds required for toxicology and clinical evaluation 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.

APPLIED ANALYTICAL INDUSTRIES (NO1-CM9-7571)

This resource contract provides the DCT 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 NCI for subsequent redistribution to clinical investigators.

ARTHUR D. LITTLE, INC. (NO1-CM8-7284)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the Contractor through a Task Assignment mechanism. These studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) *in vitro* stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following 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 is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Pre-clinical 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. This contract is scheduled to expire on September 14, 1991.

ASH STEVENS, INC. (NO1-CM1-7510)

This service preparative contract provides for the resynthesis of a variety of compounds required for the preclinical toxicology and clinical evaluation 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.

ASH STEVENS, INC. (NO1-CM9-7592)

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

ATLANTIC RESEARCH CORPORATION (NO1-CM0-7313)

The major objective of this project is to perform approved Task Orders. A Task Order might conceivably involve any facet of applications programming including 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. Current tasks include development and support of the DTPMENU, CSRS, ASRS, ADM and INVIVO systems. It also maintains and services three laser printers and a microVAX in Executive Plaza North. The contract was awarded in March of 1990 for a five year period.

AUSTRALIAN INSTITUTE OF MARINE SCIENCE (NO1-CM0-7322)

This contract furnishes approximately 1000 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 two-year contract was initiated in November, 1989, and over 1,000 organisms have been collected from Australia, Thailand and Papua New Guinea. Collections have recently begun in the Philippines.

BATELLE MEMORIAL INSTITUTE (NO1-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 (NO1-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 (NO1-CM9-7617)

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

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

This Contractor is required to manufacture parenteral dosage forms of compounds with potential anti-HIV activity. Compounds are selected and provided by the Government. The Contractor is responsible for manufacturing these dosage forms in conformity to U.S. FDA current Good Manufacturing Practice regulations. Other responsibilities include quality control testing, packaging, labeling and distribution of the final product to the NCI. This contract is being recompeted. The new project will provide for the manufacturing of anticancer and anti-AIDS agents.

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

This resource contract provides for the development and production of parenteral clinical dosage forms of anticancer agents. The Contractor has the capability of preparing production batches of liquid-filled and lyophilized sterile products. Specifically, the Contractor performs the following services: (1) formulation development of parenteral products; (2) production of sterile products; and (3) quality assurance testing of finished products. All products are packaged, labeled, and shipped to the NCI for subsequent redistribution to clinical investigators. This contract is currently being recompeted. The new project will provide for the manufacturing of anticancer and anti-AIDS agents.

CALBIOMARINE TECHNOLOGIES, INC. (N43-CMO-7358) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Contractor is investigating the large-scale agriculture of the marine bryosoan, Bulgula neritina, as a source of the antitumor agent, bryostatin. Strains of the organism collected from various locations have been cultivated, and several have been shown to produce bryostatin. Work is proceeding on scaling-up the process and imporving the yields of the bryostatin. This contract expired March 27, 1991.

CAPITOL TECHNOLOGY INFORMATION SYSTEM (N01-CM0-7353)

This contract supports the design, coding, and implementation of the biology database system. During the previous year, CTIS developed several new systems to support the biological screening effort. These include: (a) development of a system to support the activities of the Biological Evaluation Committee; (b) development of a system to support the anti-HIV syncytial screen; an interactive test assignment system; (c) auxiliary systems for support of the fraction screen and combination drug in-vitro studies; and (d) various tracking and reporting systems. This contract was awarded March of 1990 for a five year effort.

CAPITAL TECHNOLOGY INFORMATION SYSTEM (N01-CM9-7600)

This contract provides for the maintenance of the DTP AIDS Screening Database. Ongoing activities include: (a) managing the Avail/Assign System which automates the drug selection process for testing and is subsequently used to ensure that valid data are returned; (b) error Management System which is used to track and report on all the erroneous data received; (c) the Update/Edit System which can now perform referential checks of the test results against the assignment on a daily basis; and (d) several application programs such as the Supplier Reports, the Graphical Plate Analysis Report, the Tracking Report and the Plate Editor. This contract was awarded in February of 1989 for a five year period.

CHARLES RIVER LABORATORIES (NO1-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 expan- sion colonies which support the production colonies. All colonies are main-tained in a barrier environment. Offspring from the production colonies are used for hybrid production and the many NCI research activities.

ERC BIOSERVICES CORPORATION (N01-CM0-7317)

This Contractor furnishes the NCI 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.

ERC BIOSERVICES CORPORATION (NO1-CMO-7320)

This resource contract provides the DCT 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 maintains a complete computerized inventory management and drug accountability system. Recently, an increasing proportion of this Contractor's effort has been focused on performing blinded labeling and packaging of clinical trial materials for randomized NCI-sponsored studies.

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 the Pharmaceutical Data Systems (PDS). These systems are large computerized systems designed to support the many aspects of the 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 these systems; 2) detection and corrections of any problems within the systems; and 3) conversion of the various components to the ORACLE Relational Database management system.

FEIN-MARQUART ASSOCIATES, INC. (NO1-CM9-7068)

This contract provided support in the areas of computer software design, programming, implementation and, maintenance to the DTP. The primary thrust was in the area of the Natural Product Repository Support System (NPRSS). The NPRSS was written under this contract, converted from a DEC-10 based system to a VAX 8820 based system, and then converted to an ORACLE-based system. This contract was awarded in February 1989 and was closed out in February 1991.

GEORGIA, UNIVERSITY OF (NO1-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 is developed is used to verify the stability of NCI's investigational drugs during the clinical trials and is supplied to the U.S. Food and Drug Administration in support of NCI's IND filings. This Contractor also has the responsibility of conducting reserve sample inspections as required by the U.S. FDA Current Good Manufacturing Practices. This contract is currently being recompeted.

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: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that posess elements of both congener and prodrug; and (c) to synthesize compounds related to products of natural origin and other related heterocycles. These products may include partial structures of analogs and novel heterocycles. This contract is under recompetition.

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 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.

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

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

HARLAN/SPRAGUE-DAWLEY, INC. (NO1-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 expan- sion colonies which support the production colonies. All colonies are main-tained in a barrier environment. Offspring from the production colonies are used for the many NCI research activities.

HAUSER CHEMICAL RESEARCH (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 (N01-CM9-7554)

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. Assignments for the extraction and isolation of taxol (NSC 125973) from the bark of Taxus brevifolia have recently been completed.

HAWAII, UNIVERSITY OF (N01-CM6-7745)

The goals of this Contract are: the cryogenic culture of 900 cyanobacteria over a five-year period; preparation of extracts from the above mentioned cyanobacteria; the delivery of cryopreserved cultures to the NCI repository; and 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 (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.

HIPPLE CANCER RESEARCH CORPORATION (N43-CMO-7361) (SMALL BUSINESS INNOVATION RESEARCH Program)

This Phase I SBIR contract examined the feasibility of generating standardized predictive data on human and murine myelotoxicity from an *in vitro* bioassay. To do this it was necessary to replace purified cytokines and/or conditioned media with recombinant cytokines, optimize these assays of quantitative measurement of myelotoxicity, and develop a murine model to assess value and validity of the *in vitro* assay to predict *in vivo* toxicity.

Twelve drugs were looked at under this contract. Although these data were developmental in nature they did demonstrate that for at least 7 of the agents (2'3'-Dideoxycytidine, 2'3'-Dideoxyadenosine, 2'3'-dideoxyinosine, Buthionine sulfoximine-Melphalan, Hepsulfam, Pyrazine Diazohydroxide and Fostriecin) the assigned starting doses and schedules were not absolutely cytotoxic to the committed stem cells. It appeared from this work that the murine and human cells were equally sensitive to these agents *in vitro*. This contract expired March 27, 1991.

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 NCI for extraction and testing in the anticancer and AIDS-antiviral screens. To date, over 8,000 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 which have appeared in the world literature which 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 has been phased out.

ILLINOIS, UNIVERSITY OF (NO1-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.

IOWA, UNIVERSITY OF (NO1-CM9-7572)

This resource contract provides the DCT 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 NCI for subsequent redistribution to clinical investigators.

IOWA, UNIVERSITY OF (NO1-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 NCI. 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 NCI for redistribution to clinical investigators. This contract is currently being recompeted. The new project will provide for the manufacturing of anticancer and anti-AIDS agents.

KANSAS, UNIVERSITY OF (NO1-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. This contract is currently being recompeted.

LELAND B. STANFORD, JR., UNIVERSITY OF (NO1-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 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 completed the task of large-scale culturing of the cyanobacteria, Lyngbya lagerhemii and Phormidium tenue, as sources of active sulfolipids.

MARTEK 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 659 protozoans are in culture of which 535 are in axenic culture. Extracts from over 410 strains have been provided to the NCI for testing.

MARTEK CORPORATION (N43-CM0-7359) (SMALL BUSINESS INNOVATION RESEARCH Program)

This contract studied the feasibility of obtaining sulfolipids from a microalga (MK 8909) present in the Martek collections. The Laboratory of Drug Discovery Research and Development has carefully examined the extract provided by Martek Corporation and reports the following: "There appears to be little, if any, of the desired sulfolipid components described for further study as potential anti-HIV agents in this crude extract." This contract expired March 27, 1991.

MAYO FOUNDATION (NO1-CM9-7618)

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

MIAMI, UNIVERSITY OF (NO1-CM1-7533)

This contract provides for a complete pathological, parasitical, and microbio-logical 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 Offcier.

MIDWEST RESEARCH INSTITUTE (NO1-CM7-3713)

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

resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the Contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring. This contract is currently being recompeted.

MIDWEST RESEARCH INSTITUTE (NO1-CM8-7202)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 30 days and pharmacokinetics. The data from these studies are used to prepare attachment 6a to an INDA. This is the fourth year of a five year contract.

MIDWEST RESEARCH INSTITUTE (NO1-CM8-7228)

Midwest Research Institute is one of the two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the 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 (NO1-CM1-7534)

This contract provides for a complete pathological, parasitical, and microbio- logical 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 (NO1-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 sub- tropical 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 6,500 samples have been collected from Cameroon, Central African Republic, Gabon and Tanzania, and collections are proceeding in Madagascar.

NATIONAL ACADEMY OF SCIENCES (NO1-CM0-7316)

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

NEW MEXICO, STATE UNIVERSITY OF (N01-CM8-7278)

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

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 the NCI for extraction and testing in the anticancer and AIDS-antiviral screens. To date, over 6,000 samples have been collected from 13 countries, including over 500 medicinal plants used by traditional healers in Belize.

NORTH CAROLINA, UNIVERSITY OF (NO1-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. This contract is currently being recompeted.

NORTHWESTERN UNIVERSITY (NO1-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 (NO1-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 (NO1-CM9-7619)

Preclinical pharmacology studies of antitumor agents under development of DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data, as well as the efficiency of the Phase I trials of new agents. Task Assignments are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the

number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was recompeted and awarded for three years on April 30, 1989.

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

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

PHARM-ECO LABORATORIES, INC. (NO1-CM1-7543)

This service preparative contract provides for resynthesis of a variety of compounds required for preclinical toxicology and clinical evalution 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 devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities.

PHARM-ECO LABORATORIES, INC. (NO1-CM9-7587)

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

PHARM-ECO LABORATORIES, INC. (NO1-CM9-7590)

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

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 recently completed working on a task to isolate Taxol, NSC 125973, from the bark of Taxus brevifolia.

PROGRAM RESOURCES, INC. (NO1-CO7-4102)

This Contractor is located at the Frederick Cancer Research and Development Center (FCRDC) in Frederick, Maryland. The Contractor is responsible for receiving, identification, storage, and development of natural product material from sources throughout the world. As requested, these products are extracted and prepared for testing. Natural product and synthetic materials are tested, as directed by NCI for in vitro activity against 60 human tumor cell lines and for in vitro protection against the AIDS virus. Agents of further interest are tested for in vivo activity against selected human tumors and for in vivo protection against the AIDS virus. Data from in vitro and in vivo testing is collected, processed, and analyzed. Human tumors are collected

and developed as replacement cell lines for the current panel. Renovation support is provided for all DTP projects at FCRDC. Quality Control support is provided for the Animal Production and distribution program. Murine and human tumors are distributed to qualified investigators as directed by NCI. Visiting scientists from third world countries are provided laboratory support.

PURDUE RESEARCH FOUNDATION (N01-CM6-7699)

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

PURDUE RESEARCH FOUNDATION (N01-CM8-7268)

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

RESEARCH TRIANGLE INSTITUTE (N01-CM7-3714)

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

RESEARCH TRIANGLE INSTITUTE (N01-CM8-7227)

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

RESEARCH TRIANGLE INSTITUTE (N01-CM9-7561)

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

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 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.

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 which 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 of DTP are conducted under this contract to collect data which will be used to improve both the interpretation of preclinical toxicology data, as well as the efficiency of the Phase I trials of new agents. Task Assignments are issued to contractors to perform defined pharmacologic projects. In general these studies are conducted in parallel with or prior to preclinical toxicology evaluations of the experimental agents. It is the expectation that from these studies will come assays of antitumor agents of sufficient sensitivity to be used in the

clinic. Further studies will be designed to investigate the pharmacokinetics of the drug after bolus and infusion dosing in mice, rats and/or dogs. A major objective of this project is the collection of data which will ultimately lead to an understanding of species differences so that a significant reduction in the number of doses may result in the dose escalation schemes employed in Phase I clinical trials. The pharmacological information obtained through the Task Orders will play a potentially greater role in the drug development process in the future because of the increasing emphasis on in vitro screens which is contemplated in the next several years. Basic pharmacokinetic data will contribute to the planning of treatment regimens and when significant species differences are observed preclinically, the Phase I clinician is alerted to their potential human significance. This is a Task Order Managed contract which was recompeted and awarded for three years on April 30, 1989.

SOUTHERN RESEARCH INSTITUTE (NO1-CM0-7315)

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 involve murine tumors growing in pathogen-free immune-competent mice. A diversity of non-routine in vivo experiments are designed and conducted to optimize antitumor activity and evaluated 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 (NO1-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.

SOUTHERN RESEARCH INSTITUTE (NO1-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 which are no longer available from the original sources. The majority of assignments involve the preparation of structures for which detailed experimental procedures are available. This contract is under recompetition.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7235)

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

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7237)

This contract "Primary Screening of Compounds Against HTLV-III/LAV (Human AIDS Virus)" provides a capacity of 20,000 tests per annum. It is used for the testing of pure synthetic compounds and is the primary vehicle to obtain this testing. The contract has assumed increased responsibility for confirmatory testing of agents selected from the primary screen, current capacity 40 tests per annum, and for range-of-action testing with various isolates of HIV-1 and HIV-2. This is the third year of a five year contract.

SOUTHERN RESEARCH INSTITUTE (NO1-CM8-7259)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 30 days and pharmacokinetics. The data from these studies are used to prepare attachment 6a to an INDA. This is the fourth year of a five year contract.

SOUTHERN RESEARCH INSTITUTE (NO1-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 immuno-deficiency 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 (NO1-CM8-7285)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the Contractor through a Task Assignment mechanism. These studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) in vitro stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following 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 is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Pre-clinical 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. This contract is scheduled to expire on September 14, 1991.

SOUTHERN RESEARCH INSTITUTE (NO1-CM9-7553)

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

SOUTHERN RESEARCH INSTITUTE (NO1-CM9-7574)

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

SPRINGBORN RESEARCH INSTITUTE (NO1-CM8-7256)

This is a contract for the preclinical toxicological evaluation of potential antineoplastic drugs. Studies are performed in both rodents and dogs in order to determine target organ toxicity and its reversibility and to recommend safe clinical starting doses. The types of studies performed are: single and multiple dose toxicity; toxicity after continuous administration of up to 30 days and pharmacokinetics. The data from these studies are used to prepare attachment 6a to an INDA. This is the fourth year of a five 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 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.

SRI INTERNATIONAL (NO1-CM6-7864)

SRI International is one of the two contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the 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 (NO1-CM7-3715)

SRI International is one of the three contractors responsible for the chemical analysis of bulk chemicals and clinical formulations which are to be investigated in the pharmacological, toxicological, and clinical phases of the Developmental Therapeutics Program as potential antitumor agents. The Contractor determines identity and purity of the compounds by appropriate methods. The Contractor also determines solubility, stability and other physical-chemical properties to aid in

formulation development and selection of storage conditions. Techniques commonly used include elemental analysis, chromatography (thin layer, gas liquid and high performance liquid), spectroscopy (ultraviolet, infrared and nuclear magnetic resonance), gas chromatography/mass spectrometry, and other methods as needed. Reports of the work performed by the Contractor provide the data to be included in IND filings with the Food and Drug Administration and for quality assurance monitoring. This contract is currently being recompeted.

STARKS ASSOCIATES, INC. (N01-CM0-7341)

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.

STARKS ASSOCIATES, INC. (N01-CM1-7544)

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

STARKS ASSOCIATES (N01-CM8-7231)

This contract is 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 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. This contract is under recompetition.

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

contract is currently being recompeted. The new project will combine production for preclinical toxicology and for all phases of clinical trials.

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

This contract is in support of the Drug Synthesis and Chemistry Branch's (DSCB) 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 NCI'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 DSCB 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 & III trials as potential anti-AIDS agents. The compounds prepared are not readily available from the original supplier or commercial sources in the amounts and/or quality required. The major thrust of the effort of this contract is devoted to the preparation of large quantities of materials, in the multikilogram range, requiring pilot plant facilities. This contract is currently being recompeted. The new project will combine production for preclinical toxicology and for all phases of clinical trials.

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

STATE UNIVERSITY OF NEW YORK RESEARCH FOUNDATION (NO1-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: (a) to synthesize congeners of synthetic compounds with confirmed activity; (b) to design and synthesize prodrugs, and other compounds that posess elements of both congener and prodrug; and (c) to synthesize compounds related to products of natural origin and other related heterocycles. These products may include partial structures of analogs and novel heterocycles. This contract is under recompetition.

TACONIC FARMS (NO1-CM5-7730)

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

TECHNICAL RESOURCES, INC. (NO1-CM8-7249)

This DTP contract provides a program-wide resource for support services to the extramural preclinical anticancer and anti-AIDS drug discovery and development efforts. The services include: (1) support to the functions of decision-point committees; (2) planning and logistical management for DTP-sponsored conferences, seminars, and workshops, including preparation of proceedings; (3) maintenance of files for the grants, contracts, and 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 (NO1-CM3-7536)

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

UTAH, UNIVERSITY OF (NO1-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 that is being applied to resolve difficult formulation problems. This contract is currently being recompeted. The new project will provide for dosage form development for both anticancer and anti-AIDS agents.

VERMONT REGIONAL CANCER CENTER (N01-CM8-7286)

This contract provides a resource for pre-clinical pharmacology investigations of anti-AIDS agents under development by DTP. Defined pharmacological studies are assigned to the Contractor through a Task Assignment mechanism. These studies may include (1) development of sensitive analytical methodology for determination of compounds in biological fluids; (2) *in vitro* stability and protein binding studies; (3) determination of pharmacokinetic profiles and derived parameters following 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 is used to determine the most appropriate route and schedule of administration for achieving sustained viral inhibitory concentrations of the agent in biological fluids. Pre-clinical 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. This contract is scheduled to expire on September 14, 1991.

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 has completed an assignment to recollect 13 lichen species for further study as sources of potential anti-HIV agents.

Z, INC. (N01-CM7-3720)

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

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 for the clinical application of hyperthermia. This contractor is expected to continue to implement and conduct such a program and make the scientific community aware of the need for quality assurance 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 contract to assess the state-of-the-art of radiation therapy and the quality of patient care in the U.S. The Patterns of Care Study (PCS), which runs to October 1993, will extend our knowledge about patient outcome as a function of the radiation therapy 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 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 for educational programs designed to improve the quality of patient care and to document best current management for specific tumors and disease sites.

AUCKLAND UNISERVICES LIMITED (NO1-CM0-7321)

The objective of this contract is to design, synthesize and characterize new and novel hypoxic and aerobic radiosensitizers. Compounds with wide range of actions will be synthesized and tested both *in vitro* and *in vivo* for radiosensitizing activity.

CALIFORNIA, UNIVERSITY OF (NO1-CM9-7315)

The University of California, Los Angeles, (UCLA) is a member of the Neutron Therapy Collaborative Working Group (NTCWG), a consortium of three institutions funded by the NCI to carry out neutron therapy clinical trials. UCLA participated in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy, which were completed in early Spring 1991. The contract will continue at a reduced level through 1993, to support data management and follow-up of treated patients.

LASERTHERAPEUTICS, INC. (N44-CM0-7261)

This contractor is developing a NdYag (Neodymium-Yitrium-Aluminum-Garnet) laser-based system for interstitial hyperthermia for the treatment of cancer. It would provide local hyperthermic heating which would not have constructive/destructive interference problems and which can be easily monitored and controlled by the use of an inexpensive microthermal couple system. The system will provide a local hyperthermic condition that can be used as an adjunct to other therapeutic modalities, i.e., chemotherapy, radiation therapy and photodynamic therapy.

MASSACHUSETTS, UNIVERSITY OF (NO1-CM9-7570)

The University of Massachusetts, Worcester, MA, was awarded a contract in June 1989. This was in response to an RFP (NCI-CM5-7744) 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 biotin derivatives radiolabeled with In-III and To-99m 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.

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

This contractor is part of a collaborative effort which the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded from 1989-94 to develop computer-based support systems that will provide new software tools to make three-dimensional treatment planning for radiation therapy a routine activity. Automatic extraction of anatomical features from multiple CT images for the construction of three-dimensional volumes, and new tools that assist in the selection of optimal treatment plans are among the new developments to come from this effort. 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 during 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. This contract will terminate at the end of FY 91.

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

This Phase II SBIR contract is developing a real-time, portal imaging device which can produce a good quality image within the first few seconds of a radiation therapy treatment using solid-state photovoltaic sensors. The feasibility study funded under Phase I optimized performance of the cadmium-telluride (CdTe) detectors in the high energy, high intensity environment of a photon beam being used for radiation therapy. The 40-cm linear array of CdTe sensors scans through a radiotherapy port at a rate of about 10 cm/sec, and captures the data to a personal computer. The product from this SBIR contract would give radiation therapy departments the capability to capture portal images verifying patient position within a few seconds and continue the verification process during treatment in real-time.

TECHNISCAN, INC. (N44-CM9-7625)

This company is developing an advanced ultrasound imaging system for application to the diagnostic imaging of abdominal organs and the breast in human subjects. The system consists of a multiple transducer, multi-channel adaptive array, and image processing and display systems. By developing specific algorithms based on principles of synthetic focus, inverse scattering, adaptive array, and other imaging principles, it is anticipated that spatial resolution in ultrasound images can be improved to a fraction of one millimeter. Progress in this development has been followed by computer simulation imaging at various stages. Procurement, fabrication, and evaluation of components and subsystems has been proceeding well in accordance

with a PERT-planned management and scheduling system. Theoretical and applied activities have been undertaken in collaboration with the Department of Engineering at the University of Utah in support of this development.

TEXAS, UNIVERSITY OF (NO1-CM5-7775)

The University of Texas System Cancer Center, M.D. Anderson Hospital (MDAH) 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 participated in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy, which were completed in early spring 1991. The contract will continue at a reduced level through 1993 to support data management and follow-up of treated patients.

WASHINGTON, UNIVERSITY OF (NO1-CM9-7282)

The University of Washington 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. Washington participated in the randomized Phase III trials comparing fast neutron therapy with best conventional radiation therapy, which were completed in early spring 1991. The contract will continue at a reduced level through 1993 to support data management and follow-up of treated patients.

WASHINGTON UNIVERSITY (NO1-CM9-7564)

This contractor is part of a collaborative effort, the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded from 1989-94 to develop computer-based support systems that will provide new software tools to make three-dimensional treatment planning for radiation therapy a routine activity. Automatic extraction of anatomical features from multiple CT images for the construction of three-dimensional volumes, and new tools that assist in the selection of optimal treatment plans are among the new developments to come from the effort. Code and software documentation developed by the RTPT-CWG will be published in the public domain for widest possible dissemination to the radiotherapy community.

WASHINGTON, UNIVERSITY OF (NO1-CM9-7566)

This contractor is part of a collaborative effort, the Radiotherapy Treatment Planning Tools Collaborative Working Group (RTPT-CWG) funded from 1989-94 to develop computer-based support systems that will provide new software tools to make three-dimensional treatment planning for radiation therapy a routine activity. Automatic extraction of anatomical features from multiple CT images for the construction of three-

dimensional volumes, and new tools that assist in the selection of optimal treatment plans are among the new developments to come from the effort. Code and software documentation developed by the RTPT-CWG will be published in the public domain for widest possible dissemination to the radiotherapy community.

SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE BIOLOGICAL RESPONSE MODIFIERS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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) and with the Cancer Therapy Evaluation Program (CTEP) of the Division of Cancer Treatment (DCT). A group of individuals comprised 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 activites 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 variables, 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 L. 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 John R. Ortaldo, Ph.D. 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 efforts 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 107 grants with nearly $29 million, including 77 research projects (R01), 8 program projects (P01), 1 outstanding investigator grant (R35), 4 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 FY91 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. In addition, in FY91 a new program announcement entitled "Specific Cancer Cell Targeting Using Molecular Genetic Technology" was issued in conjunction with the Developmental Therapeutics Program.

SUMMARY OF BRB ACCOMPLISHMENTS IN FY91

The extramural program has: maintained 24 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 monoclonal antibodies for clinical studies of imaging and radiotherapy; expanded the distribution of biological standards to include IL1α, IL1β, IL2, IL3, IL4, IL6, IL8, tumor necrosis factor (TNF)-α, TNF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), transforming growth factor (TGF)-α, TGF-β; awarded $5.4 million for extramural contracts, $2.7 million in clinical trials and $2.7 million for preclinical contracts; begun planning the development of a pilot plant for the production of clinical grade monoclonal antibodies and other recombinant molecules at NCI-FCRDC.

New Extramural Initiatives

The BRB is in the process of developing Requests for Proposals (RFPs) in the following areas: production of chimeric human-mouse monoclonal antibodies, recompetition of the BRM repository, establishment of Master Agreements to support innovative investigator-initiated clinical trials involving complex or sophisticated immunological manipulations, and establishment of Master Agreements for the large-scale production of clinical grade cancer-specific targeting agents. 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./DynCorp 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. The PEL has demonstrated a significant diurnal variation in the toxicity of chemotherapy. The maximum tolerated dose of carboplatin, a myelotoxic platinum analogue, is 130 mg/kg when administered to mice at 9:00 AM. On the other hand, the maximum tolerated dose is about 200 mg/kg when administered at 9:00 PM. Experiments designed to evaluate the basis of this difference are ongoing; however, it does not appear that there are major differences in the pharmacokinetics of an administered dose of the drug at the two times of day. Instead, there seems to be a distinct threshold of susceptibility to the toxic effects, perhaps related to the circadian variation in cell cycling of marrow stem cells. Efforts are now underway to evaluate the antitumor effects of the higher doses of drug administered in the evening.

The increased use of colony-stimulating factors has raised the question of whether repeated cycles of stimulation of marrow stem cells followed by cytotoxic chemotherapy is leading to stem cell depletion. The PEL treated mice with six cycles of cyclophosphamide, an alkylating agent with relative stem cell-sparing activity compared to other alkylating agents. Animals were also given either pretreatment with IL1 or restorative therapy with G- or GM-CSF or were given both pretreatment and restorative treatment. Animals were then evaluated for the damage done to the stem cell compartment by performing measurements of stem cell function in vitro and serial bone marrow transplantation in vivo. Animals that received cyclophosphamide therapy with G- or GM-CSF support had significantly greater reduction in CFU-S and long-term marrow repopulating activity than animals treated with cyclophosphamide alone. Furthermore, the use of IL1 pretreatment appeared to ameliorate the enhanced toxicity of the cyclophosphamide plus G- or GM-CSF regimens. Thus, a major concern about using G- or GM-CSF together with cyclic combination chemotherapy regimens is the potential for enhancing damage to the stem cell compartment. The use of IL1 together with G- or GM-CSF may prevent this damage.

Although bone marrow toxicity is dose-limiting for many chemotherapeutic agents, other organ toxicities are also noted at high doses. For example, though carboplatin produces dose-limiting myelotoxicity, it also produces significant gastrointestinal tract toxicity that can be detected by the presence of diarrhea clinically in mice and the presence of microscopic changes in the bowel wall. IL1 has been found to protect mice from fatal toxicity associated with high-dose carboplatin toxicity. The IL1 appears to act not only at the level of hematopoiesis, but the PEL now has evidence that the gastrointestinal tract toxicity is also ameliorated by IL1. The data on IL1 protection against carboplatin toxicity has led to a clinical trial in which the effects of IL1 on carboplatin toxicity in humans is being assessed.

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 cosponsored with CTEP an international meeting on the use of IL2 in hematologic malignancies held in Bethesda on September 12-13, 1991. The BRMP also sponsored a number of Therapeutic Applications Group meetings to review progress to date on IL8, IL4, IL1, IL3, and interferon-γ. In addition, 31 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 is reviewed and discussed. There are regular meetings of the scientists and the clinicians called Therapeutic Applications Group meetings organized around particular areas of science from which clinical applications are developed. There is abundant scientific exchange in weekly staff meetings and monthly laboratory and branch chiefs meetings. 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. Other research in the laboratory is being done by William J. Murphy, Ph.D., a post-doctoral fellow, and in collaboration with scientists at Program Resources, Inc./DynCorp. Dr. Ashwell has been investigating the molecular mechanism of the two major forces that shape the T-cell repertoire, namely positive and negative selection. In normal T cells, activation through the T-cell antigen receptor leads to T-cell proliferation and cytokine production. In T-cell hybridomas, receptor-mediated activation also results in cytokine production, but also leads to cell death by apoptosis. Corticosteroids also lead to apoptosis. However, the two stimuli that lead to apoptosis act by quite distinct mechanisms. Death induced by T-cell receptor activation is blocked by cyclosporin A, but steroid-induced death is not. Conversely, steroid-induced death is blocked by RU-486 but receptor-mediated death is not. Simultaneous exposure to both antigen and steroids prevents cell death. It appears that the glucocorticoid receptor prevents the binding of the AP-1

transcription factor (which is induced by antigen activation) from binding to its DNA binding site. In addition, AP-1 appears to compete with the glucocorticoid receptor for binding to a composite glucocorticoid response element (GRE). Thus, the mutual interference of the two death signals is related to specific blocking of the action of stimulus-specific transcription factors. These data lead to a testable model for the basis of negative and positive selection in thymocytes. Negative selection occurs when either a thymocyte interacts avidly with a thymic MHC or MHC-peptide complex because such a signal leads to cell death. Similarly, negative selection occurs if there is no interaction between a T-cell receptor and the thymic environment because of the unopposed steroid effect. However, when the T-cell antigen receptor is engaged in an environment where glucocorticoid is also present, apoptosis is blocked and positive selection occurs. This model is under investigation.

Dr. Kruisbeek and her colleagues have been among the world's leaders in dissecting the role of the thymus in the development of the T-cell repertoire. Her group has demonstrated the important role of the cytokines IL2 and IL4 in T-cell development. Furthermore, they have shown that $CD4^+$, class II MHC-restricted T cells require cognate cell interactions in the thymus between T cells expressing CD4 and thymus cells expressing class II MHC molecules and that $CD8^+$, class I MHC-restricted T cells require cognate cell interactions in the thymus between T cells expressing CD8 and thymus cells expressing class I MHC molecules. Recently, this group has demonstrated that blocking cell adhesion molecules, such as ICAM-1, can interfere with normal T-cell development. The in vivo physiology is being further investigated in vitro by means of an ingenious cell culture method in which undifferentiated thymocyte cell lines (transformed with a recombinant v-myc and v-raf-containing retrovirus) are exposed to thymic epithelial cell lines and undergo differentiation. This simple model involving two clonal cell lines should permit dissection of the signals that pass between thymic epithelial cells and thymocytes which lead to T-cell development. In recognition of her international stature in the immunology community, Dr. Kruisbeek has just been recruited to serve as Chairman of the Department of Immunology at the Netherlands Cancer Institute in Amsterdam. We are most disappointed that she will be leaving the BRMP but are pleased that her outstanding work has led to her being offered such an important scientific leadership position.

Dr. William Murphy, an extremely capable post-doctoral fellow, has been conducting research on both the basic science and preclinical levels involving transplantation biology. In his basic studies, he has devised a new experimental model for studying the early events in T-cell development. Using mice with severe combined immunodeficiency (SCID) which have a defect in their ability to rearrange their immunoglobulin genes and their T-cell receptor genes, he has found that exposure of these mice to low doses of irradiation resulted in the induction of differentiation with the expression of CD3 on the cell surface which, on immunoprecipitation, appears to be associated with a normal appearing α/β T-cell receptor heterodimer. Studies on the signal transduction through the radiation-induced T-cell receptor are underway. However, this model provides a unique opportunity to study the molecular events relating to T-cell receptor gene rearrangement. A second major project involves the administration of human lymphoid cells to SCID mice, the creation of human-mouse chimeras. Such animal models are important in the study of AIDS, autoimmunity and tumor immunology but have been impeded by the unstable

and unpredictable level of chimerism. Dr. Murphy has found that administration of human cells together with human growth hormone resulted in much improved human T-cell engraftment including the detection of human T cells in the murine thymus. Studies with growth hormone-deficient mice have revealed that growth hormone is an essential thymopoietic hormone and can greatly augment immune reconstitution after lethal irradiation and bone marrow transplantation. One of the important basic science questions that this work will address is the degree of plasticity of the T-cell repertoire. Will the human T-cells developing in a SCID murine thymus learn to recognized murine MHC molecules as self? Of more direct clinical relevance is his work on bone marrow transplantation which predicts that the use of human growth hormone would greatly accelerate the recovery of T-cell function. Further studies have identified a critical role for natural killer (NK) cells in allogeneic bone marrow transplantation. Dr. Murphy has found that the administration of donor-type IL2-activated NK cells prevents graft-vs-host disease, speeds hematologic engraftment, and promotes immune recovery. He is now using these adoptively transferred activated natural killer (NK) cells as a component of antitumor therapy in tumor-bearing hosts. Preliminary data suggest that NK cells produce a variety of cytokines that promote hematopoietic and lymphoid cell development. This work has major potential to make allogeneic bone marrow transplantation a safer and more effective form of cancer treatment.

In collaboration with James J. Kenny, Ph.D., of Program Resources, Inc./DynCorp, the laboratory is also conducting studies on B-cell development. Using transgenic mice expressing either heavy chain immunoglobulin genes, light chain immunoglobulin genes, or both, several important ideas have emerged. First, the basis for the selection of the antigen-specific B-cell repertoire has been examined. Normally, the murine response to the antigen phosphocholine, an important component of bacterial cell walls, is dominated by an antibody bearing the T15 idiotype. By inserting a heavy chain gene of the M167 idiotype into the germline, we have found that all of the phosphocholine-specific B cells in such transgenic animals are M167 idiotype positive. Thus, B cells in which the transgene encoded H-chain has associated with an endogenous Vλ24 light chain have been specifically amplified. T15 idiotype positive cells are not detected in these mice. However, antibodies composed of the M167 heavy chain and the Vκ22 light chain, are T15 positive but cannot bind to phosphocholine. Since T15 idiotype positive B cells should be generated in such transgenic mice, but are not selected for expansion during development, these experiments are the first to suggest that the selection of the B-cell repertoire is driven by antigen rather than by anti-idiotype networks, as hypothesized by Jerne. A second major finding is that B cells expressing both the M167 heavy and light chain genes that bind to phosphocholine are triggered to expand and proliferate in normal mice; however, when such cells also express the xid genetic defect, environmental or autologous, phosphocholine stimulates apoptosis and cell death.

In collaboration with Margaret Beckwith, Ph.D., of Program Resources, Inc./DynCorp, the laboratory is investigating the mechanism of activation-induced cell death in human lymphoma cell lines. Dr. Beckwith has demonstrated that exposure of an IgM-expressing human B-cell lymphoma cell line to antibody directed against IgM (which should mimic exposure of the cell to antigen) results in irreversible growth arrest. The process is independent of phosphoinositide metabolism and protein kinase C activation but is dependent upon tyrosine phosphorylation. The two major substrates of

phosphorylation after exposure to anti-IgM are proteins of 112kD and 86kD molecular weight whose identities are under investigation. Growth arrest is promoted by inhibitors of phosphatase activity (such as vanadate and okadaic acid) and reversed by inhibitors of tyrosine kinase activity (such as herbimycin A and genistein). Thus, the accumulation of phosphorylated proteins is associated with growth arrest. The lymphoma cell line, RL, which demonstrates this response also possesses a mutant form of p53, which decreases in cells that are growth arrested. Similarly, transforming growth factor-β augments the growth inhibition mediated by low concentrations of anti-IgM antibody; it also speeds the disappearance of mutant p53. The role of mutant p53 in lymphoma biology is unclear. Most untreated lymphomas that have been examined do not have mutant p53. We have three cell lines from previously treated patients, all of which bear mutant p53. Our single lymphoma cell line from a previously untreated patient has wild type p53. We are cloning and sequencing the mutant p53 genes to see if there is a particular mutation that occurs in patients exposed to chemotherapy. We are also exposing the lymphoma cell line with wild type p53 to chemotherapy in vitro to see if we induce mutations in p53.

Chou-Chi Li, Ph.D., of Program Resources, Inc./DynCorp has extended her studies on the *mos* oncogene. In addition to its more widespread distribution in dividing cells than previously thought, she has shown that the mos protein is associated with tubulin and with cdc2. In view of its role to promote cell division, *mos* may be an important therapeutic target. Even more interesting than her work with *mos* is her demonstration that HTLV-I appears to produce cell transformation indirectly by activating the transcription factor *rel*. She has shown that *rel* expression is induced by the *tax* gene product and it appears that a 40kD *rel* protein may bind with the *tax* gene product to form a novel transcription factor that directs the induction of proliferation-related genes. These data resolve the paradox of HTLV-I transformation in the absence of a transforming gene.

Douglas K. Ferris, Ph.D., of Program Resources, Inc./DynCorp has continued his studies of the regulation of cell cycle progression. Using antibodies directed against distinct portions of cdc2, he has demonstrated that cdc2 is complexed with different proteins in distinct cellular compartments and in different phases of the cell cycle. It appears that the cellular distribution of cdc2 and its associated proteins is regulated at least in part by its state of phosphorylation. Dr. Ferris has identified the major mammalian tyrosine kinase that inactivates cdc2 and has begun to characterize a phosphatase that may activate cdc2 by dephosphorylation.

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., Scott Durum, Ph.D., and Louis Matis, M.D., along with a new senior staff member who is not yet tenured, David Kelvin, Ph.D.

Dr. Oppenheim has been studying the effects of cytokines on the sensitivity or resistance of hematopoietic cells to radiation or chemotherapeutic agents that generate free radicals. He and his colleagues have found that antibodies to

the IL1 receptor and anti-TNF antibodies completely abrogated the radioprotective effects of IL1 as well as those of endotoxin. TGF-β was also found to be radiosensitizing, possibly by downregulation of IL1 receptors. The mechanism of the radioprotective effects of IL1 were further studied at the cellular level. Cells transfected with manganese superoxide dismutase (MnSOD), a mitochondrial membrane protein induced by IL1, became more radioresistant and conversely, cells transfected with antisense cDNA for MnSOD were sensitized to radiation as well as drugs such as doxorubicin and mitomycin C. These data suggest that the level of MnSOD determines the degree of protection induced by IL1. Dr. Oppenheim has also been examining the role of cytokines in the promotion and inhibition of carcinogenesis. TNF has been shown to be similar to phorbol esters in its capacity to promote anchorage-independent growth, soft agar colony formation and tumorigenesis in the JB6 epithelial cells line. In contrast, TGF-β blocked these effects and synergized with retinoids in the prevention of transformation.

Dr. David Kelvin has embarked on an ambitious project to clone the IL8 and MCAF receptors. These receptors for cytokines defined and cloned at the BRMP are important in the inflammatory response and are prototypical of a new class of receptors called the chemotactic intercrine family. The physical nature of the receptors has been studied and defined but the genes have not yet been cloned.

Dr. Scott Durum has been studying the effects of IL1 in T-cell activation. Building on his previous work demonstrating that IL1 induced c-jun and the formation of AP-1 when the IL1-induced c-jun combines with antigen-induced c-fos, he has demonstrated that IL1 leads to activation of preformed AP-1 complexes and stabilization of c-jun mRNA. Dr. Durum has also developed an in vitro technique for studying the regulation of the signals that lead to rearrangement of T-cell receptor Vβ genes. In an effort to study the role of IL1 in vivo, he and his colleagues have embarked on a high-risk project aimed at knocking out IL1 genes in embryonic stem cells by homologous recombination.

The recruitment of Dr. Louis Matis, an internationally known cellular immunologist, has greatly added to our expertise in T-cell biology. Dr. Matis is well known for his work relating the structure of particular antigens to the generation of antigen-specific receptors made from particular V gene segments. He demonstrated that administration of bacterial superantigens to mice in vivo led to Vβ-specific tolerance that was produced both through induction of clonal deletion and clonal anergy. This work could lead to important treatments for autoimmune diseases mediated by autoreactive T cells such as multiple sclerosis, juvenile diabetes, and other serious illnesses. He has also led the efforts to define the biology and function of the subset of T cells expressing $\gamma\delta$ rather than $\alpha\beta$ heterodimer antigen receptors. Using transgenic mice expressing a particular $\gamma\delta$ receptor pair specific for a defined class I MHC molecule, he found that such cells could not exit the thymus to repopulate the periphery in mice in which β2-microglobulin expressing was blocked. Since β2-microglobulin expression is required to express class I MHC molecules on the cell surface, these data are interpreted to mean that $\gamma\delta$ T cells require receptor-mediated signalling in order to differentiate and leave the thymus.

The Immunobiology Section under Dr. Luigi Varesio has focussed on the biology of monocytes and macrophages. He and his colleagues have found that IL2 is a potent activator of monocytes that results in the induction of IL6 production, M-CSF receptor and IL2 receptor-α chain expression, and the development of tumor cytotoxic activity. IL2 effects can be augmented and prolonged by costimulation with IFN-γ or M-CSF. In contrast, IL4 and TGF-β inhibit IL2 activation. Studies of the molecular mechanisms of these cytokine interactions are in progress. George W. Cox, Ph.D., in the Immunobiology Section has been studying gene expression in immortalized murine macrophages. In contrast to human normal monocytes, neither IL2 alone nor IFN-γ alone are capable of activating the cells to kill tumor cells. However, costimulation of the macrophage lines by IL2 plus IFN-γ produces potent tumoricidal activity as does lipopolysaccharide (LPS) plus IFN-γ. IL4 inhibits IL2 plus IFN-γ-induced cytotoxicity, but not LPS plus IFN-γ-induced cytotoxicity. Dr. Cox has also identified the induction of genes by IFN-γ. In addition to the induction of several genes encoding chemotactic cytokines, two novel genes are also induced which are being characterized. This work is beginning to shed light on the regulation of gene expression of cells of the monocyte/macrophage lineage.

The Lymphokine Section under Dr. Frank Ruscetti studies the regulation of hematopoiesis by cytokines and the regulation of gene expression by human retroviruses. Dr. Ruscetti and his colleagues have demonstrated that TGF-β1 and TGF-β2 are equipotent inhibitors of proliferation of early progenitors with TGF-β3 being about 10 times more potent than the other members of the family. The effects of TGF-β are directly mediated on early progenitors and are related to the trans-down modulation of cell surface receptors for stimulatory cytokines and interference with post-receptor signalling of the stimulatory cytokines. The inhibitory effects of TGF-β extend to neoplastic cells of hematopoietic lineage, as well. TGF-β is potent at inhibiting the development of cytotoxic T cells and acts at least in part by blocking the expression of the IL2 receptor α-chain. A surprising finding was the bidirectional effects of TGF-β on hematopoiesis. There is a myeloid cell committed progenitor that is synergistically stimulated by TGF-β plus GM-CSF. This newly defined precursor, called BFU-G, preserves granulopoiesis when TGF-β is administered in vivo to mice. Ruscetti and coworkers have also done important work on the regulation of viral replication of the human retroviruses, HTLV-I and HIV. He and his colleagues have shown that a cellular protein present in human B cells but not in human T cells is capable of suppressing retroviral transcription. Provirus is integrated by viral proteins are not expressed. However, certain stimuli (such as irradiation and protein kinase C activation) permit activation of viral transcription such that the virus can be passed to uninfected normal human B and T cells. The nature of the protein responsible for the inhibition of viral expression is being studied. Similar studies in an HIV system have demonstrated that macrophages, a natural reservoir for HIV, also can suppress HIV expression beginning 4-6 weeks after infection. The inhibition of HIV expression in monocytes appears to be mediated by the inhibition of binding of NF-κB to the HIV enhancer. Such studies may lead to strategies for eliminating viral latency in man.

The Cytokine Molecular Mechanisms Section under Dr. William Farrar is trying to analyze the specific pathways of activation of cells by cytokines. He and his colleagues have found that several cytokines, including IL2, IL3, GM-CSF

and erythropoietin, appear to have very similar effects upon the cells that respond to them. The receptors for these cytokines do not appear to contain sequences with intrinsic kinase activity, but each of them mediate tyrosine kinase activity, presumably through a kinase that associates with the receptor protein. Dr. Farrar and his colleagues have undertaken site-specific mutagenesis of the erythropoietin receptor and have defined the regions required for growth promotion and coupling to the kinase. They have also identified several new kinases, one of which, designated RLK, appears to be related to the c-fms protooncogene. Recent work conducted by Diana Linnekin, Ph.D., in this laboratory has suggested that a p97 protein may be a candidate tyrosine kinase that is common to all hemopoietin receptors.

Laboratory of Experimental Immunology (LEI)

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

The Leukocyte Cell Biology Section under the acting head, Dr. Ortaldo, has made major progress in understanding the basis by which NK cells recognize tumor targets. They first made an antibody to an NK target cell (K562) that blocked the binding of NK cells to tumor targets and then made an anti-idiotype to that target-specific antibody that recognizes an NK cell surface molecule. The anti-idiotypic antibody appears to recognize the NK antigen receptor. A cDNA clone of the molecule recognized by the anti-idiotype has been sequenced and it appears to be a unique protein. They have mapped the gene to chromosome 3 and have been studying the impact of 3p deletion in Von Hippel-Lindau syndrome on NK activity. It was this group's recognition of the fact that NK cells responded to IL2 in the absence of expression of the p55 IL2 receptor α-chain that led to the identification of the p75 high affinity IL2 receptor β-chain. They have also been studying the effects of IL2 on the production of a major pore-forming protein (perforin) thought to be involved in the cytolytic mechanism. IL2 directly augments levels of perforin mRNA in T cells, particularly of the $CD8^+$ subset. In contrast, IL2 made no impact upon the high constitutive expression of perforin mRNA in CD3-negative NK cells. Thus, the effect of IL2 to broaden the target specificity and augment the killing of NK cells is not related to an increase in production of lytic proteins. One of the most surprising and interesting observations made by scientists in this section involves studies of NK cell ontogeny. They have shown that a thymocyte (the so-called dull Lyl-positive cell) can give rise to hepatic large granular lymphocytes upon adoptive transfer. This is the first evidence that links NK cell development to T-cell development and brings forward the level of differentiation of their common progenitor.

The Cellular and Molecular Immunology Section, under Dr. Howard Young, studies the control of gene expression during the development and maturation of the cellular immune response. They have led the way in understanding the regulation of IFN-γ gene expression. Two enhancer elements for the IFN-γ gene have been identified, one of which is tissue-specific. The tissue-specific enhancer is located 5' to the coding sequence and also contains a repressor region. The second enhancer is located in the first intron.

The Experimental Therapeutics Section is led by Dr. Robert Wiltrout. A major emphasis of the laboratory has been the development of cytokine regimens capable of permitting an increase in the maximum tolerated dose of a chemotherapeutic agent in an animal model. They generated the first data suggesting that a particular dose and schedule of IL1 permitted a 40% increase in the dose of cyclophosphamide and such increased doses were significantly more effective in tumor-bearing animals than the previously determined maximum tolerated dose without cytokine administration. Application of these results to man is ongoing. The group has also begun to study the role of IL7 in boosting the immune system in vivo. Together with Kristin Komschlies, Ph.D., of Program Resources, Inc/DynCorp, they have shown that IL7 produces and expansion in early B and T cells, stimulates some antigen-dependent T-cell functions, and induces LAK activity. Adminstration of IL7 produces a profound decrease in stem cell numbers in the bone marrow and a dramatic increase in stem cell numbers in the spleen. Mice treated with IL7 develop a leukocytosis consisting predominantly of pre-B cells and CD8-positive T cells. In therapeutic models of experimental metastases, IL7 is active as a single agent. However, perhaps its greatest promise is in the promotion of the development of T-cell specific tumor immunity. Preliminary animal data are encouraging. Drs. John Pearson and William Fogler are trying to develop effective therapies to overcome the multidrug resistance phenotype. They have demonstrated in vivo efficacy of antibody to the p170 glycoprotein at restoring sensitivity to vincristine in an mdr-expressing human colon cancer xenograft model. Similar effects have been observed with quinidine. They have recently generated an immunotoxin based upon the anti-p170 antibody conjugated to a toxin called verrucarin A. Preliminary in vitro data are encouraging that such an immunotoxin may be an effective way to combat the development of drug resistance. Pearson and Fogler are also pursuing methods of enhancing the in vivo killing effects of immunotoxins. They have demonstrated substantial synergy in antitumor effects when IFN-α is administered together with an immunotoxin. Studies are currently in progress to define the basis of the synergistin interaction. Anne Pilaro, Ph.D., in the laboratory has been studying the chemotactic activity of NK cells. IL2, IL6 and TNF stimulate directed migration of NK cells, but IL1, IL8, IFN-γ, and TGF-β do not. Migration and penetration of NK cells into peripheral organs involves attachment to endothelial cells. Dr. Pilaro has established in vitro models that examine the impact of NK cell products on the expression of cell adhesion molecules on endothelial cells. In collaboration with Thomas Sayers, Ph.D., of Program Resources, Inc./DynCorp, the laboratory has been characterizing a novel protein from the granules of NK cells. The protein induces growth inhibition, rounding up of the cell morphology, and homotypic aggregation of target cells from a variety of species. The protein was purified to homogeneity, characterized as a 29-31 kD protein and its sequence was found to be identical to a recently cloned serine protease designated RNKP-1.

The Laboratory of Biochemical Physiology (LBP)

The Laboratory of Biochemical Physiology (LBP) is directed by Hsiang-fu Kung, Ph.D. This laboratory 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's group has been instrumental in working out the physiology of the ras oncogene. Ras proteins are G-proteins, meaning that they have GDP/GTP binding activity and have GTPase activity. Oncogenic ras has considerably less GTPase activity than cellular ras proteins. An important means of regulating ras function is the exchange of GDP bound to ras (inactive form) with GTP, which results in ras activation. Dr. Kung and his colleagues have identified a protein that they call guanine nucleotide exchange factor (rGEF) which activates inactive ras. It is conceivable that a therapeutic strategy aimed at inhibiting rGEF could be of value in tumor types that depend upon active ras.

Dr. Robert G. Fenton has been collaborating with Dr. Kung's group in the area of immunizing animals against tumors transformed by mutant ras oncogenes. He has successfully induced tumor-specific immunity against tumors transformed by the immunizing mutant form of ras but not crossreactive immunity to tumors transformed with other mutant ras forms. In view of the prominent role of ras activation in pancreatic cancer, adenocarcinoma of the lung, and colon cancer, it is possible that this strategy may lead to a vaccine that may be effective in ras-mediated tumors.

A very important finding from the Kung laboratory deals with the discovery of a new mechanism of transformation. Kung and his colleagues have found that microinjection of eukaryotic initiation factor-4E (eIF-4E) promoted DNA synthesis and morphological cell transformation. Coinjection of protein kinase C amplified these effects and coinjection of neutralizing antibody to the ras oncogene inhibited the transformation. Protein kinase A appears capable of phosphorylating eIF-4E and blocking its transforming capacity.

The laboratory has been examining signal transduction pathways in Xenopus oocytes. In order to better understand later stages of embryogenesis, they have developed an embryo tissue culture system to study differential participation of ventral and dorsolateral mesoderms in the hemopoiesis of the developing frog. Addition of TGF-betal to such stem cell cultures promotes hematopoiesis in ventral mesodermal tissue. Further studies on the control of organ development and the role played by particular signal pathways in differentiation are planned.

Dr. Michael Schwabe has been attempting to prove that a 110kD IL6-binding molecule on the surface of myeloma cells and certain other tumors is a high affinity IL6 receptor. Data are still unclear on this point. However, he has shown that IL6 can mediate growth inhibition of certain tumor cell lines that express the receptor.

The LBP has also been studying AIDS. In collaboration with Sylvia Lee-Huang, Ph.D., of New York University, they have developed several single-chain ribosome inactivating proteins (including MAP30, TAP29, GAP30, DAP30, and DAP32) from Chinese medicinal plants that appear to be capable of inhibiting HIV infection and replication, viral core protein p24 expression, and HIV reverse transcriptase. The in vitro therapeutic index appears to be quite large with these compounds. Efforts are beginning to bring one of these

agents to clinical trial. The laboratory has also been identifying cellular factors from monocytes that bind to the negative regulatory element of HIV. Several novel DNA binding proteins have recently been cloned.

Clinical Research Branch (CRB)

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 lymphoproliferative diseases. 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. There are currently 22 active protocols. The CRB entered about 300 patients onto studies in FY91.

Early in the clinical development of IL2, it appeared that there was a relationship between the induction of LAK activity in the peripheral blood and the probability of response. However, we have now established administration doses and schedules of IL2 that augment peripheral blood LAK activity in 100% of patients; but this has not led to an increase in the response rate. We have attempted to augment the response rate from IL2-based therapy by adding cyclophosphamide, polyICLC, or flavone acetic acid. Each of these studies demonstrated an augmentation in immune function in treated patients and each agent appeared to improve the immune functions normally augmented with IL2; however, they have not led to an impressive increase in antitumor effects. Nevertheless, there is the possibility that some combination of these agents will permit similar immunomodulation at lower doses of IL2 with less IL2-related toxicity.

One of the most important findings of the past year has been the impressive response rate we have obtained in patients with malignant melanoma in response to IL2 plus the monoclonal antibody, R24. R24 is a murine IgG3 antibody that can fix human complement and mediate a human inflammatory response. In its phase I and II development as a single agent, it has produced responses in about 20% of patients. It recognizes the GD3 ganglioside expressed on melanoma cells and, interestingly, it has recently been shown that GD3 is expressed on a fraction of T cells. In an effort to augment its antitumor effects, we combined R24 with IL2 in two protocol designs. In the first protocol, the R24 was given first and three days later followed by high dose boluses of IL2, similar to those used in LAK plus IL2 studies. Since imaging studies generally show that the best tumor:normal tissue ratio of an antibody occurs after 3 days, we reasoned that the antibody would have time to home to the tumor and then IL2-activated NK cells bearing Fc receptors would recognize antibody-bound tumor cells and kill them. The second strategy was to build up the number of Fc receptor-bearing cells by delivering IL2 in a 24-hour infusion twice weekly. We have previously shown that patients so treated develop a prominent lymphocytosis mainly comprised by activated NK cells. The idea was that more tumor killing might occur if there were more activated NK cells to mediate the killing. There have been no responses on the first protocol; the response rate on the second protocol has been about 45%, roughly

twice the response rate we have previously seen with IL2 alone. Most importantly, responders could be separated from nonresponders by the appearance in their peripheral blood of IFN-γ. Responders and nonresponders could not be separated on the basis of the levels of NK cells, NK activity, or LAK activity that they attained. Further studies are underway to better understand the basis for the production of IFN-γ in the responding patients and to increase the fraction of responders.

IL2 was combined with IFN-α in an outpatient regimen. The response rate in renal cell carcinoma was 33%.

In an effort to use activated T cells as adoptive immunotherapy without the technical difficulties associated with tumor-infiltrating lymphocytes, we have been performing a phase I study of cells activated in vitro with anti-CD3, an antibody that activates the cells through the antigen receptor. Such anti-CD3-activated cells (called T-AK for T-cell activated killers) are administered to patients together with IL2. There has been considerable expansion of these activated cells in vivo with peripheral blood counts reaching 90,000/μl. Patients also developed a coagulopathy and a severe metabolic acidosis not seen in our previous IL2 plus LAK cells protocols. It appears that the acidosis occurs as a function of glycolysis in the expanded cells. Dose-limiting toxicity is hypotension, which appears to be related to the nitrate levels in the peripheral blood. Nitrates may be able to be blocked pharmacologically providing a potential method for eliminating hypotension as a dose-limiting toxicity. Biopsy of tumor sites during therapy has revealed a marked increase in the cellular infiltrate of T cells in treated patients. We are hopeful that this form of therapy will be useful in the treatment of cancer. Further cohorts of patients are being studied to optimize this therapy.

The recent success of levamisole plus 5-fluorouracil as adjuvant therapy in patients with Duke's C colon cancer led us to inquire about the in vivo immune effects of levamisole. There has been no convincing evidence to suggest that levamisole has any particular biological response modifying activity. However, our study suggested strongly that levamisole is acting at the level of the monocyte/macrophage since it can enhance the production of neopterin from such cells in vivo when administered in combination with IFN-γ. This study may lead to more rational use of levamisole in future studies.

In view of the substantial interest of the BRMP in IL1, we are extensively involved in the clinical development of IL1 as an antitumor agent, an immune stimulant, and a hematopoietic growth factor. Maximal tolerated doses of IL1α and IL1β have been determined and phase II studies are underway to evaluate IL1 in each of its potential activities. Preliminary data suggest that IL1 is capable of ameliorating the platelet toxicity of carboplatin given at doses above the maximum tolerated dose. If these preliminary findings are validated in larger numbers of patients, IL1 could be used in patients with germ cell cancer, ovarian cancer, lung cancer, and hematopoietic malignancies. Studies involving intratumoral injection of IL1, use of IL1 to support autologous bone marrow transplantation, and exploration of a BRMP-discovered synergy between IL1 and etoposide.

The CRB conducts a number of disease-related studies, particularly in the hematologic malignancies. In patients with hairy cell leukemia treated with IFN-α, we were the first to detect the occurrence of neutralizing anti-IFN antibodies as a source of treatment resistance. Further follow-up of patients developing antibodies revealed that the antibody did not persist. Resistant patients retained responsiveness to alternative forms of IFN-α and the neutralizing antibodies disappeared with the return of responsiveness to the IFN-α2a with which they were originally treated. We have also shown that patients kept on IFN-α continuously for prolonged periods continue to have improvement in the disease response and their hematologic parameters continuously throughout the treatment period. We were the first group to combine IFN-α and deoxycoformycin in the treatment of hairy cell leukemia. All treated patients remain in remission off all therapy for over 4 years. This is the first evidence for persistence of response off therapy. Patients treated with deoxycoformycin developed rather severe decreases in $CD4^+$ T-cell counts as a consequence of therapy. Nearly all the patients make at least some recovery of their CD4-positive T-cell counts over the first 3 years after treatment; however, many do not return to normal levels. Fortunately, these patients do not appear to develop serious opportunistic infections as a consequence of the low cell counts, but there has been an increased incidence of localized Herpes zoster.

The CRB also conducts studies in the treatment of lymphomas and Hodgkin's disease in collaboration with the Radiation Oncology Branch and the Medicine Branch. This year we published the first prospective randomized trial demonstrating the superiority of one doxorubicin-containing regimen (ProMACE-CytaBOM) over another (ProMACE-MOPP). Since six drugs are common between the two regimens, we hypothesized that the major reason for the advantage to ProMACE-CytaBOM was related to delivering the drugs at an increased dose intensity. In an effort to further improve the dose intensity of the regimen, we deliver therapy every week, instead of 2 weeks out of three. To date, the complete response rate is about 90% with only 17% relapses. Toxicity has been acceptable. The drugs have been safely delivered with significantly higher dose intensity than standard ProMACE-CytaBOM. We are hoping to interest a cooperative group in undertaking a prospective randomized study between standard and short-course ProMACE-CytaBOM.

Patients with advanced stage indolent lymphoma are being entered on a prospective randomized study comparing a conservative management approach (watch and wait or radiation to symptomatic masses) vs aggressive combination chemotherapy with ProMACE/MOPP flexitherapy followed by low dose total nodal radiation therapy. The complete response rate for patients randomized to aggressive therapy is 76% with the median complete response lasting 8 years. For patients who cross over to aggressive therapy after conservative management fails, the complete response rate is significantly lower (40%). So far, the longer disease-free survival of the aggressively treated patients has not translated into a survival advantage. Accrual to this study is about to be terminated in favor of a new treatment approach; long-term follow-up will be necessary to complete the comparison of the conservative and aggressive management approaches.

Patients with early stage Hodgkin's disease are being randomized to receive either extended-field radiation therapy (standard therapy) or MOPP combination chemotherapy. Fifty of 52 patients treated with radiation therapy achieved a

complete response and 38% of the complete responders have relapsed. In contrast, only 7 of the 55 MOPP-treated patients have relapsed. The projected 10-year disease-free survival is 60% for radiation therapy-treated patients and 86% for MOPP-treated patients (P2=0.009). Ten-year overall survival is 76% for radiation therapy-treated patients and 92% for MOPP-treated patients (P2=0.051). For patients with stage I and II A or B disease, the treatment outcome is comparable between the two modalities. Thus, the greater acute toxicity and gonadal toxicity of chemotherapy must be weighed against the toxicity of staging laparotomy, thyroid dysfunction, and development of solid tumors in patients treated with radiation therapy plus the leukemia risk for the radiation relapsers requiring salvage chemotherapy.

Patients with advanced stage or poor prognosis Hodgkin's disease are receiving dose-intense MOPP chemotherapy supported with intercycle GM-CSF. To date, 10 of 12 patients have achieved a complete response and one has relapsed. No patients have died and the population of patients has received a significantly higher dose intensity of the MOPP drugs than is delivered with conventional dose MOPP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09290-06 OAD

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.)*

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:	C. Zacharchuk	Medical Staff Fellow	OAD, BTS, NCI
	S. Volarevic	Visiting Associate	OAD, BTS, NCI
	C. Burns	Visiting Fellow	OAD, BTS, NCI
	B. Niklinska	Visiting Fellow	OAD, BTS, NCI
	Y. Yang	Visiting Fellow	OAD, BTS, NCI

COOPERATING UNITS *(if any)*

Merck Sharp & Dohme (F. Dumont), Immune Cell Biology Program, Naval Medical Research Institute (H. Yamada, C. June)

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:
4.0	4.0	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.)*

T-cell hybridomas and thymocytes undergo programmed cell death (apoptosis) when stimulated with reagents that are normally mitogenic. They also die when stimulated with glucocorticoids such as dexamethasone (Dex). Interestingly, the combination of cellular activation and Dex results in cell survival. The mechanism of this mutual antagonism may be at the level of nuclear transcription factors. Dex treatment prevents the induction of AP-1 (a Jun-Jun or Jun-Fos dimer). Conversely, T-cell receptor (TCR)-mediated activation prevents gene transcription enhanced by a newly described composite glucocorticoid response element. This may represent a novel mechanism for receptor "cross-talk" in intact cells. It also provides the basis for a new model that deals with thymic selection by proposing a balance between TCR-mediated programmed cell death (negative selection) and its antagonism by corticosteroids (positive selection). Other experiments have explored the function of the CD45 tyrosine phosphatase. CD45 has been shown to be physically associated with the TCR. This association has now been mapped to the δ and ζ chains, the later being of particular interest because it itself is tyrosine phosphorylated after activation. Analysis of CD45 variants that express varying levels of this molecule demonstrated a direct inverse correlation between CD45 levels and spontaneous phosphorylation of ζ. There was also an excellent direct correlation between CD45 levels and TCR-mediated phosphoinositide hydrolysis and Ca2+ flux. Surprisingly, even in the absence of CD45 there were TCR-induced late Ca2+ oscillations. Thus, some but not all components of TCR coupling to Ca2+ regulation is CD45 (tyrosine phosphatase) dependent.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Investigator	OAD, BTS, NCI
Charles Zacharchuk	Medical Staff Fellow	OAD, BTS, NCI
Sinisa Volarevic	Visiting Associate	OAD, BTS, NCI
Christopher Burns	Visiting Fellow	OAD, BTS, NCI
Barbara Niklinska	Visiting Fellow	OAD, BTS, NCI
Yili Yang	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. Activation- and Steroid-Induced Cell Death

Both glucocorticoids and T-cell receptor (TCR)-mediated stimulation cause programmed cell death (often referred to as apoptosis) in T cells. We have previously shown that for T-cell hybridomas the actions of these two lethal stimuli are mutually antagonistic. That is, treatment with dexamethasone (Dex) prevents the killing induced by antigen (or anti-TCR antibodies), and vice versa. Some notable features of this antagonism are:

1. Cyclosporin A (CsA) blocks activation-, but not Dex-, induced death.
2. When used as antagonists, either stimuli can be added up to four hours after the other and still block cell death.
3. TCR-mediated activation prevents the death of normal T-cell clones.
4. RU-486 blocks Dex-, but not activation-, induced cell death.
5. There is no antagonism at the level of steroid receptor translocation to the nucleus or steroid-driven transcription of steroid enhancer-regulated reporter gene.

Other groups have recently shown that the dimeric transcription factor AP-1 (consisting of either Jun-Jun or Jun-Fos) can physically bind the glucocorticoid receptor (GR), resulting in the alteration of function of both factors. To determine if such an interaction could account for the phenomenon of mutual antagonism of programmed cell death, a number of experimental approaches were employed.

The 2B4.11 T-cell hybridoma was transiently transfected with a plasmid consisting of a CAT reporter gene 3' of 5 tandemly repeated TRE's (TPA Response Elements, or AP-1 binding sites). Various means were used to stimulate these cells, and CAT activity assessed by TLC. First, phorbol ester induced a very large amount of CAT activity. Stimulation with an anti-TCR antibody (2C11) induced about 20% as much CAT activity, consistent with its

induction of moderate levels both Fos and Jun (as judged by Northern blot analysis). Dex did not induce CAT activity. Interestingly, stimulation with 2C11 in the presence of Dex resulted in a substantial (> 60%) decrease compared to 2C11 in medium alone. A similar result was obtained for PMA in the presence or absence of Dex. To confirm that this actually reflected an interaction between the GR and AP-1, gel shift assays were performed using an AP-1 oligonucleotide. As expected, both phorbol ester and 2C11 induced a protein (AP-1) that caused this oligonucleotide to shift its migration in an agarose gel. Dex did not induce such a factor. Dex did, however, prevent both the phorbol ester- and the 2C11-induced increase in AP-1. These data support a model in which the bound GR interferes with the interaction of AP-1 with its DNA binding site. Since AP-1 appears to play an important role in regulating IL-2 secretion, this may account for the inhibitory effect of steroids on IL-2 production. Moreover, it may implicate AP-1 as having a role in signalling for activation-induced programmed cell death.

As discussed above, cell activation did not prevent steroid-induced CAT activity driven by a classic glucocorticoid enhancer (a glucocorticoid response element, or GRE). Classic, or simple, GREs consist of a 15 nucleotide consensus sequence and positively regulate the transcription of numerous genes. In the past several years a number of new, nonhomologous, GREs have been described that either positively or negatively regulate gene transcription. These GREs have been called negative GREs (nGREs), complex GREs, or composite GREs. One such nGRE, found 5' of the proliferin gene, has been shown to either positively or negatively regulate gene transcription, the determining factor in influencing the outcome apparently being the presence or absence of the AP-1 transcription factor. This nGRE was found to have two cryptic AP-1 binding sites, possibly accounting for the modification of its activity by AP-1. We tested an nGRE-CAT construct in 2B4.11 cells. Surprisingly, in the absence of any specific stimulus there was a small but significant amount of CAT activity. Dex caused a 60% increase in the basal activity. Of particular interest was that activation with 2C11 inhibited both the basal CAT activity and the Dex-induced activity. Phorbol ester had the same qualitative effect as 2C11, although it was considerably more potent. Gel shift analysis with the nGRE oligonucleotide was then performed. Nuclear extracts from unstimulated 2B4.11 cells caused the shift of a discrete band. This shift was blocked by cold nGRE competitor but not by the cold classic GRE oligonucleotide. Finally, phorbol ester induced a second gel shifted band that co-migrated with an AP-1 oligonucleotide. Thus, unstimulated 2B4.11 cells express a nuclear binding factor (which is not the GR) that binds the nGRE.

Together, these data support a role for an nGRE (composite GRE) in steroid-induced programmed cell death. In this model TCR-mediated activation induces AP-1, which is necessary but not sufficient to cause cell death. The ligand-bound GR binds AP-1, preventing activation-induced death. Conversely, the activated GR binds an nGRE, which results in the transcription of one or more genes that are necessary for cell death. AP-1 also binds this nGRE, but in such a way that it prevents GR binding without any agonist activity of its own. This model will be tested by transfecting AP-1 into 2B4.11 cells and assessing its effect on Dex-induced CAT transcription and programmed cell death.

In a second series of experiments, the activity of the *bcl-2* gene has been analyzed. This gene, initially identified in human B-cell leukemias, has been shown to prolong the life of normal B cells, and in transgenic animals leads to B cell tumors. *bcl-2* is currently the only known gene whose product is widely believed to cause resistance to programmed cell death. To determine what effect, if any, *bcl-2* would have on activation- or steroid-induced T-cell death, 2B4.11 cells were transfected with *bcl-2*. The expression vector has a neomycin (*neo*) resistance gene, and contains an SFFV (spleen focus forming virus) promoter and 5' regulatory sequence. Two independent transfectants have been analyzed at this time. Both express the *bcl-2* product, as judged by immunoblotting. Both transfectants die normally when stimulated with 2C11 or antigen. However, the cells have an intriguing phenotype when stimulated with Dex. That is, concentrations of Dex up to 3×10^{-6} M cause the usual dose-dependent programmed cell death. Higher concentrations, however, result in a dose-dependent decrease in cell death. This phenomenon is not seen with *neo* only transfectants. Inspection of the SFFV sequence revealed that it has a classic GRE. Reasoning that Dex may actually enhance *bcl-2* levels, immunoblots were performed on *bcl-2* transfectants that had been incubated with high levels of Dex for 4 to 8 hrs. Dex induced a 3- to 10-fold increase in *bcl-2* levels. The working hypothesis at this time is that *bcl-2* antagonizes steroid-mediated, but not activation-induced, programmed cell death. Interestingly, Dex still antagonizes activation-induced cell death, suggesting that the lethal effects of glucocorticoids and their ability to antagonize TCR-mediated death are separable.

II. Physical Associations Between Biologically Active T-Cell Molecules

We have previously shown that the CD45 molecule, a transmembrane tyrosine phosphatase, is intimately associated with Thy-1 and the TCR. The association with the TCR is especially provocative because the TCR ζ chain is itself phosphorylated on tyrosines after cellular activation. Attempts have been made to determine if the TCR association is chain-specific and, if so, is ζ involved. Two approaches have been used. First, 2B4.11 T cells were surface iodinated and cross-linked with the cleavable homobifunctional reagent DSP. The cells were lysed, the lysate immunoprecipitated with anti-TCR antibodies, and the labeled material resolved by SDS-PAGE. Only one potential TCR chain was seen on these gels, a band with a Mr of 16 kd which could represent ζ. To ensure the identity of the co-precipitated species, we obtained antisera or monoclonal antibodies against all of the TCR chains, and have performed Western blot analysis of CD45 co-precipitated material from chemically cross-linked cells. The results of such an analysis revealed that precipitation of CD45 did not bring down TCR α, β, γ, or ϵ. In contrast, large amounts of both ζ and δ were co-precipitated. Identical results were obtained using digitonin lysates of untreated (uncross-linked) 2B4.11 cells. CD45 also co-precipitated a truncated form of ζ (truncated at residue 87 out of a total of 143). It is not clear at this time if CD45 associates primarily with either δ or ζ, or if it associated equivalently with both. Recent work from others has suggested that it is only the TCRs that are occupied by ligand that have ζ chains that are phosphorylated on tyrosines. Experiments are currently underway to determine if an association with CD45 confers this property.

Another approach to the role of CD45 in T cells has been to generate T-cell clones that express varying levels of this molecule. Such cells have been made in collaboration with Francis Dumont, who used ethyl methanesulfonate and repetitive cell sorting to obtain subclones of the YAC-1 T-cell line that express levels of CD45 ranging from normal (wild type, WT) to negative (clones N1 and N2). The levels of CD45 mRNA correlate with the level of CD45 surface expression. Furthermore, the levels of anti-CD45 precipitable tyrosine phosphatase activity (the enzymatic function of CD45) correlate perfectly with both surface protein and mRNA levels. All of the cells are TCR^+, $CD4^-$. $CD8^-$, $Thy\text{-}1^+$, and $Ly\text{-}6^-$.

To assess the effect that CD45 loss might have on the tyrosine phosphorylation status of the TCR in these cells, anti- phosphotyrosine immunoblotting was performed on anti-TCR immunoprecipitates resolved by SDS-PAGE. In WT cells the level of ζ phosphorylation went from barely detectable in resting cells to a major band after 2C11 activation. When cells with a progressive loss of CD45 were analyzed, there was evident a steady increase in baseline ζ phosphorylation. In fact, in $CD45^-$ cells the level of ζ phosphorylation was equivalent to that of WT cells stimulated with 2C11. These data suggest that one role of CD45 is to maintain ζ in an unphosphorylated state. They also suggest that the normal state of ζ phosphorylation is due to the balance of a constitutively active tyrosine kinase and the CD45 tyrosine phosphatase.

Two classic signalling pathways are phosphoinositide (PI) hydrolysis and Ca^{2+} flux. There was an excellent direct correlation between these two parameters and CD45 expression. In collaboration with Carl June and Hidehiro Yamada, we have pursued the Ca^{2+} signalling events with a relatively new imaging technique that lets one follow individual cells over time. Surprising, and in contrast to the Ca^{2+} data obtained by flow cytometry, anti-TCR stimuli did cause changes in Ca^{2+} levels in the $CD45^-$ cells, although in an unusual way. There was no rapid mobilization of Ca^{2+}, as was seen in the WT cells. Rather, 4 to 5 minutes after stimulation there was the appearance of individual cell Ca^{2+} oscillations. Experiments are now underway to determine how these late oscillation relate to Ca^{2+} flux in the WT cells. Regardless, these data prove that only some aspects of TCR signal transduction are controlled by CD45, and demonstrate novel CD45-independent Ca^{2+} changes.

SIGNIFICANCE

Signals from the environment regulate the growth, differentiation, and behavior of T cells. How these signals are "translated" to cellular responses is one of the major mysteries in immunology today. The observation that cellular activation via the TCR and exposure to corticosteroids, both of which independently cause programmed cell death, are mutually antagonistic raises the interesting question of how these two apparently parallel pathways interact with one another. Our preliminary results suggest that this interaction occurs at the level of nuclear transcription factors. Thus, TCR-induced AP-1, which is important for IL-2 production and, perhaps, cell death appears to prevent steroid induction of genes dependent upon a special, complex, glucocorticoid response element. Similarly, steroids prevent the induction (or binding) or AP-1 to its target nucleotide sequence. In addition

to providing a model for transcription factor "cross-talk", these results may provide clues as to how occupancy of these receptors leads to programmed cell death. Another issue is how the TCR couples to any cell activation pathway. CD45, the most abundant tyrosine phosphatase in T cells, has recently been implicated as a prerequisite for the normal sequelae of T-cell activation. Our data suggest that the situation is more complicated than that. First, loss of CD45 results in spontaneous phosphorylation of the TCR ζ chain. This provides one potential means by which CD45 might regulate TCR signalling. Although TCR-mediated phosphoinositide hydrolysis and Ca^{2+} flux are deficient at a gross level, even in the absence of CD45 stimulation via the TCR causes individual cells to manifest repetitive Ca^{2+} oscillations. This unexpected observation suggests that the TCR has more than one way it can regulate Ca^{2+} levels. Future work will include analysis of CD45 as a "co-signalling" molecule by reconstituting $CD45^-$ T cells with mutated forms of the CD45 gene.

PUBLICATIONS

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Zacharchuk CM, Mercep M, Chakraborti PK, Simons SS Jr, Ashwell JD. Programmed T lymphocyte death: cell activation- and steroid-induced pathways are mutually antagonistic. J Immunol 1990;145:4037-4045.

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Zacharchuk CM, Mercep M, Ashwell JD. Thymocyte activation and death: a mechanism for molding the T cell repertoire. In: Edelson R, Ashwell JD, eds. Antigen- and Clone-Specific Immunoregulation. New York: New York Academy of Sciences, 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09310-05 OAD

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.)*

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
	P. McQuillen	Howard Hughes Fellow	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. We found that both interleukin-2 (IL-2) and BSF-1/IL-4 are produced upon activation of fetal thymocytes through Thy-1 and CD3-activation. We have therefore produced transgenic mice with a disability in IL-2 production and are currently exploring early T-cell development in such mice. 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; and blocking of the IL-2R results in arrested T-cell development also in this model. Finally, a panel of early fetal thymic cell lines has been derived by v-myc/v-raf transformation, 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.

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
Patrick McQuillen	Howard Hughes Fellow	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 CD3-complex as transducers of activation signals. In addition, we demonstrated that fetal thymocytes produce both IL-2 and IL-4 upon activation. As these studies were performed at a timepoint before conventional $\alpha\beta$ receptor-bearing cells are present, the results suggest a role for early $\gamma\delta$ 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 is now 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.

PUBLICATIONS

Fine JS, Kruisbeek AM. The role of LFA-1/ICAM-1 interactions during murine T lymphocyte development. J Immunol 1991;147:2852-2859.

Kruisbeek AM, Rajewsky K. Lymphocyte development. Curr Opin Immunol 1991;3:169-170.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09311-05 OAD

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.)*

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)*

Laboratory of Cell Biology and Metabolism, NICHHD (T. Rutledge, S. Frank, R. Klausner); Experimental Immunology Section, NCI (A. Weissman); and Immune Cell Biology Program, Naval Medical Research Institute (H. Yamada, C. June)

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 availability of T-cell mutants that lack one or more of the T-cell antigen receptor 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. In contrast, the role of CD3-δ appears to be largely confined to assembly and transport of the TCR to the cell surface. The introduction of an active tyrosine kinase (encoded by v-*src*) into T cells allowed the study this signalling pathway in isolation. Although causing spontaneous IL-2 production, there was no evidence that the PI hydrolysis pathway or protein kinase C are involved. Surprisingly, intracellular Ca2+ levels were higher, and TCR-mediated Ca2+ increases were markedly enhanced in the v-*src*+ cells, suggesting the novel hypothesis that tyrosine kinases may directly regulate [Ca2+]i in T cells. In addition, the completely normal responses (PI hydrolysis, CD3-γ phosphorylation, growth inhibition, programmed cell death, IL-2 production) of these cells suggests that the theory that phosphorylation of TCR ζ desensitizes this receptor is, at best, an oversimplification. Finally, heterochimeric antibodies (made of 2 F(ab) fragments, one against the TCR and the other against an MHC class II molecule) were used to stimulate T cells with mutated TCR ζ chains. When bound to plastic, the antibodies stimulated these mutants and the wild type equally, but when "presented" by an accessory cell only the wild type responded. This is identical to the phenotype of intact, plastic-bound antibody vs. antigen. These data will be used to develop a model of how TCR occupancy initiates T-cell activation.

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, Tanya Rutledge, and Richard Klausner we have studied the biological properties of TCRs that contain mutated CD3-δ and TCR-ζ chains. First, a CD3-δ-deficient (and cell surface TCR negative) T-cell hybridoma derived from 2B4.11 was transfected with an expression vector containing a δ gene that was truncated at the location of the intracellular portion of the molecule. Although missing its intracellular portion, the product of this gene allowed complete reexpression of cell surface TCR, proving that CD3-δ is required for efficient transport and retention of the rest of the TCR chains to the cell surface. Simulation of these cells with either anti-CD3-ϵ antibodies or the antigen for which these cells are specific (pigeon cytochrome *c*) caused IL-2 production. Moreover, both the antigen dose-response curves and the amount of IL-2 produced were comparable between cells reconstituted with either full length δ or the truncated form of δ. Therefore, unlike what we have previously found with the ζ chain, intracellular δ is not required for ligand-induced signal transduction. Although these results do not rule out a role for the transmembrane or even extracellular portion of δ for this function, the simplest interpretation is that δ is required for TCR expression but does not play a direct role in TCR-mediated signalling.

The TCR ζ chain is unique in that it is phosphorylated on tyrosine residues shortly after T-cell activation. There are four internal tyrosines, all of which are thought to be phosphorylated upon activation. To determine what effect phosphorylation of any of these tyrosine residues might have on T-cell function, in collaboration with Drs. Stuart Frank, Rick Klausner, and Allan Weissman, each of the four internal tyrosines has been altered by site direct mutagenesis to a phenylalanine. These mutated ζ chains were then transfected into T-cell hybridoma mutants that lacked TCR ζ. All of the transfectants

re-expressed normal levels of the cell surface TCR. Stimulation with antigen or anti-CD3 antibodies caused various ζ phosphorylation patterns. Strikingly, the mutation of tyrosine 111 to a phenylalanine resulted in a complete lack of ζ activation-induced tyrosine phosphorylation, raising the unexpected possibility that Tyr 111 is an initial step required for subsequent tyrosine phosphorylation to occur. Regardless of the phosphorylation status of ζ, all of the subclones produced IL-2 when stimulated. Thus, phospho-ζ is not a necessary intermediate in the signalling pathway that yields lymphokine production.

II. Expression of a Constitutively Active Tyrosine Kinase in T Cells

v-*src* is a prototypic tyrosine kinase. It is constitutively active due to a truncation in a regulatory C-terminal portion of the molecule. Dr. John O'Shea introduced v-*src* was introduced into the 2B4.11 T-cell hybridoma by retroviral gene transfer, and we have collaboratively characterized these cells. v-*src*$^+$ cell were found to have many proteins phosphorylated on tyrosine in the absence of any external stimulus. Interestingly, these cells also produced IL-2 spontaneously, providing the first good model system in which tyrosine phosphorylation can directly be shown to initiate a late T-cell biological response.

We have now used these cells to explore several issues related to TCR function and T-cell signal transduction. To determine if phosphorylation of the TCR ζ chain was required for subsequent IL-2 production, ζ$^+$ and ζ$^-$ subclones of a v-*src*$^+$ T-cell hybridoma was obtained. All subclones, regardless of their ζ status, produced IL-2 spontaneously, ruling out an obligate requirement for phospho-ζ once a tyrosine kinase has been activated. There is a model of some popularity that holds that phosphorylation of ζ on tyrosine residues actually desensitizes the TCR. This is based on the *lpr* and *gld* mouse models, where the T cells have constitutively phosphorylated TCR ζ chains and respond aberrantly to activating signals. The same phenotype has recently been described in immature thymocytes. The v-*src*$^+$ cells provided us with an opportunity to explicitly test this model. Anti-TCR or antigen stimulated phosphoinositide hydrolysis, CD3-γ serine/threonine phosphorylation, growth inhibition, programmed cell death, and IL-2 production were identical amongst these cells. To rule out the possibility that we failed to detect any signalling defects in the v-*src*$^+$ cells due to a quantitative difference in the amount of phosphorylation in these cells compared to *lpr* mice, the *lpr* -/- T cells and v-*src*$^\pm$ cells were directly compared for their levels of phospho-ζ. Both populations had comparable amounts of total ζ. If anything, however, the v-*src* cells expressed more phospho-ζ than the *lpr* cells. Therefore, we conclude that phosphorylation of the ζ chain, *per se*, does not desensitize the T cell to subsequent activating signals.

In the course of the studies on signal transduction, the unexpected observation was made that TCR-mediated Ca^{2+} flux was hyperresponsive. Using fluorescence imaging to follow individual cells, it was found that three independent v-*src*$^+$ cells gave mean $[Ca^{2+}]_i$ increases of > 800 nM, while three control v-*src*$^-$ cells had responses of ~ 300 nM or less. Indeed, the resting level of $[Ca^{2+}]_i$ was approximately 2-fold higher in these cells than in the

v-*src*$^-$ control cells. Also unexpectedly, the spontaneous increase in $[Ca^{2+}]_i$, and the enhanced response to an anti-TCR stimulus, was not due to overproduction of inositol 1,4,5-trisphosphate. To determine if it was the tyrosine phosphorylation of the TCR ζ chain itself that resulted in this phenotype, ζ^-/v-*src*$^+$ cells were examined. Their phenotype was similar to that of the ζ^+/v-*src*$^+$ cells. Therefore, phosphorylation of ζ did not confer the Ca^{2+} handling abnormality. Finally, observation of the cells over time in the absence of any stimulus revealed that they have spontaneously oscillation of their $[Ca^{2+}]_i$. These results demonstrate that an active protein tyrosine kinase can markedly affect intracellular Ca^{2+} handling by a process independent of inositol 1,4,5-trisphosphate production and T-cell receptor tyrosine phosphorylation, and suggest that tyrosine kinases may normally regulate T-cell receptor-mediated changes in intracellular Ca^{2+}.

III. Using Antigen "Mimics" to Probe the Mechanism of T-Cell Activation by Antigen

We have previously described ζ^- mutants of 2B4.11 and ζ truncation mutants of 2B4.11 that express normal cell surface TCR levels, produce IL-2 when stimulated with cross-linked antibodies, but fail to make IL-2 when stimulated with antigen. The physiological basis for this is unclear. The differences between the two modes of stimulation that might matter 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 have been created. Anti-TCR α Fab fragments were chemically cross-linked to anti-MHC class II Fab fragments. Such antibodies are monovalent for both the TCR and class II molecules, and bind the two molecules that are normally involved in antigen presentation. These antibodies stimulate IL-2 production from 2B4.11 cells, but only in 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 heterochimeric antibodies (HCA). These antibodies also stimulate normal splenic T-cell proliferation.

The HCA were tested with ζ^- 2B4.11 cells that had been transfected with either full length ζ chain (FL) or a truncated ζ (CT108, a cell that does not respond to antigen). HCA composed of anti-class II coupled to anti-TCR-α, anti-TCR-β, or anti-CD3-ϵ, stimulated both cells equally well when coated on a plastic surface. However, these same three antibodies stimulated the FL subclone at approximately a 1000-fold lower concentration than required to stimulate the CT108 cell when presented by a class II-bearing cell line. Thus, the HCA have the "antigen phenotype" rather than the "antibody phenotype". These results indicate that the difference between antigen and antibody in stimulating the ζ truncated cells is unlikely to be due to affinity or conformational requirements. We hypothesize that the ζ chain is required for oligomerization of the TCR upon occupancy, a function that is perturbed by truncation. We are currently exploring the possibility that ζ is responsible for association of the TCR with the cytoskeleton.

SIGNIFICANCE

How T cells transduce signals across the plasma membrane is one of the central issues of modern immunology. The TCR's complex, multi-subunit, structure has led us to suggest that different sets of chains have different functional properties. The availability of mutant cells 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. In contrast, the role of CD3-δ appears to be largely confined to assembly and transport of the TCR to the cell surface. The introduction of an active tyrosine kinase into T cells has allowed us to study this signalling pathway in isolation. The surprising observation that Ca^{2+} mobilization was affected suggests for the first time that tyrosine kinases may directly regulate $[Ca^{2+}]_i$. In addition, this model system allowed us to determine that the theory that phosphorylation of TCR ζ desensitizes this receptor is, at best, an oversimplification. Finally, the generation of heterochimeric antibody "antigen mimics" is providing a unique insight into the mechanism underlying the initiation of T-cell activation. These novel reagents should allow us to explore issues such as TCR aggregation, cross-linking, clustering, and associations with other cell molecules and structures.

PUBLICATIONS

Frank SJ, Niklinska BB, Orloff DG, Mercep M, Ashwell JD, Klausner RD. Structural mutations of the T cell receptor ζ chain and its role in T cell activation. Science 1990;249:174-177.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09323-04 OAD

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.)*

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)*

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 has yielded important answers to the questions 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 signficance 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

Jones LA, Chin LT, Kruisbeek AM. Acquisition of self-tolerance in T cells is achieved by different mechanisms, operating both inside and outside the thymus. Thymus 1990;16:195-206.

Jones LA, Chin LT, Longo DL, Kruisbeek AM. Peripheral clonal elimination of mature functional T cells. Science 1990;250:1726-1729.

Jones LA, Chin LT, Merriam GR, Nelson LM, Kruisbeek AM. Failure of clonal deletion in neonatally thymectomized mice: tolerance is preserved through clonal anergy. J Exp Med 1990;172:1277-1286.

Rellahan BL, Jones LA, Kruisbeek AM, Fry AM, Matis LA. In vivo induction of anergy in peripheral Vβ8$^+$ T cells by staphylococcal enterotoxin B. J Exp Med 1990;172:1091-1100.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09368-01 OAD

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.)*

Acceleration of Immune Reconstitution and T-Cell Development Following BMT

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

PI:	W.J. Murphy	Post-doctoral Fellow	OAD, NCI
Others:	D.L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*

LMI, BRMP, (S. Durum); LEI, BRMP, (R. Wiltrout, J. O'Shea, H. Young); BCDP, PRI/DynCorp, (R. Hornung, J. Keller); OAD, BRMP (A. Kruisbeek); Univ. Texas Southwestern Med. Ctr., Dallas, TX (M. Bennett, V. Kumar)

LAB/BRANCH

Office of the Associate Director

SECTION

Transplantation Biology 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.)*

Bone marrow transplantation (BMT) is currently used for the treatment of various diseases yet significant obstacles still remain. These obstacles include: graft-versus-host disease (GVHD), rejection of the marrow graft by the host, and a period of immune deficiency following the BMT leaving the patient susceptible to opportunistic infections. Our work has focussed on finding ways to abrogate the host's ability to reject the marrow graft and to accelerate immune reconstitution following BMT. We have found that NK cells activated with IL-2 in vitro can produce a variety of cytokines including: TGF-β, TNF-α, GM-CSF, G-CSF, IL-1β, and a potentially novel hematopoietic growth factor as determined through RNA analysis and protein analysis of their supernatants. These NK cells could then be adoptively transferred in vivo during allogeneic BMT in mice. We found that transfer of activated NK cells syngeneic with the donor could promote marrow engraftment in allogeneic recipients. The use of these NK cells also resulted in faster immune reconstitution in the recipients and no GVHD occurred. We found that these NK cells neutralized the host effector cells and prevented rejection. Another approach was with the use of neuroendocrine hormones to accelerate reconstitution. Utilizing a model in which human lymphocytes are transferred into immunodeficient mice, we found that recombinant human growth hormone (rhGH) promoted human T-cell engraftment in these mice. Surprisingly, rhGH treatment also allowed for the entry of human cells into the mouse thymus. We have used rhGH in other murine model systems and found that rhGH exerts profound effects on T-cell reconstitution and development. We also observed that rhGH exerts significant effects on hematopoietic proliferation and engraftment following BMT.

PROJECT DESCRIPTION

PERSONNEL

William J. Murphy	Post-doctoral Fellow	OAD, NCI
Dan L. Longo	Associate Director	OAD, NCI

OBJECTIVES

1. To determine the effects of activated NK cells on hematopoiesis and bone marrow engraftment; and

2. To examine effects of neuroendocrine hormones on T-cell development and immune reconstitution.

MAJOR FINDINGS

Role of NK Cells in Hematopoiesis

Purified NK cells were obtained from mice with severe combined immune deficiency (SCID) to ascertain their effects on hematopoiesis. When activated with IL-2 in vitro, these cells maintained a phenotypic and lytic spectrum consistent with a pure population of activated NK cells. The cells were negative for CD3, CD4, CD8, T cell receptor (TCR), MAC-1, and surface immunoglobulin. The cells were positive for asialo GM1 (ASGM1) and Thy-1. When added with syngeneic bone marrow cells (BMC) in soft agar, the NK cells could support hematopoietic growth in vitro without the addition of hematopoietic growth factors. However, when syngeneic BMC were added along with exogenous cytokines to provide optimal growth conditions, the addition of NK cells were inhibitory for hematopoietic colony formation. Antibodies to interferon-γ partially reversed the inhibitory effects of the NK cells. Supernatants from the NK cells could also exert these effects on hematopoiesis, although to a much lesser extent. Analysis of the NK cells at the RNA level demonstrated that activated NK cells transcribe genes for hematopoietic growth factors such as GM-CSF, G-CSF, and IL-1. At the other end of the spectrum, the NK cells were also found to transcribe genes for interferon-γ, TGF-β, and TNF-α which could account for the inhibitory effects seen. Analysis of the NK cell supernatants using factor dependent progenitor cell proliferation assays confirmed that the NK cells were producing GM-CSF, as well as a potentially novel hematopoietic growth factor. These NK cells were used in a BMT model to determine their effects on engraftment. Activated NK cells of donor strain were then adoptively transferred with donor BMC and placed into lethally irradiated allogeneic hosts. The addition of NK cells with BMC and IL-2 allowed for greater hematopoietic engraftment as determined through short-term hematopoietic colony assays in soft agar. The use of NK cells also resulted in greater donor-derived chimerism with accelerated reconstitution of the B-cell population as determined with flow cytometric analysis phenotyping the chimeras lymphoid cells. Additionally there was no evidence of GVHD in the recipients receiving NK cells and IL-2. We have found, through the use of various strain combinations, that the NK cells were promoting engraftment at least partly through the specific neutralization of host effector cells. Thus, the use of activated NK cells may significantly augment hematopoietic engraftment and immune reconstitution in the

clinical setting of BMT. We are now applying this with a tumor model in which mice are given a syngeneic tumor and then are treated with allogeneic BMT and activated NK cells.

The Use of Neuroendocrine Mediators in Immune Reconstitution

Growth hormone has previously been implicated in T-cell development and we tested its efficacy in promoting T-lymphocyte transplantation. SCID mice were used as recipients of either murine thymocytes or human peripheral blood lymphocytes (huPBL). Injection of recombinant human growth hormone (rhGH, provided by Genentech, South San Francisco, CA) (injections were given at 5 μg intra-peritoneally for three days) strongly promoted engraftment of murine thymocytes in the lymph nodes of SCID mice as determined three weeks after transfer by flow cytometric analysis. Human T-cell engraftment was also promoted by rhGH in SCID mice, especially in the murine thymus, where no human T cells were detected unless rhGH was given. We also detected significant hematopoietic effects of rhGH. Mice given rhGH for two weeks demonstrated an increase in the hematopoietic progenitor content in their spleens and BM as determined by soft agar colony assays. Mice were then given azidothymidine (AZT) which suppresses the hematopoietic system and results in anemia. Treatment of the mice with rhGH resulted in improved hematocrit levels.

To further address the role of the neuroendocrine system in immune development, GH-deficient mice were examined. Snell-Bragg (SB) dwarf mice lack acidophilic pituitary cells and are deficient in GH production. We found that these mice exhibit decreased peripheral blood counts affecting all lineages (red cells, white cells, and platelets). Examination of their lymphoid tissues indicated that their thymus, spleen, and lymph nodes were significantly hypoplastic. Treatment with rhGH improved their peripheral blood counts and prolonged their survival. Examination of the thymus indicated there was a deficiency of CD4/CD8 double-positive progenitor cells and treatment with rhGH corrected this deficiency. Analysis of the bone marrow indicated a deficiency of $B220^+$ pre-B cells. Thus, these mice display significant myeloid and lymphoid deficiencies suggesting the GH plays a critical role in normal development. GH may be of considerable use to improve immune and hematopoietic reconstitution after BMT.

SIGNIFICANCE

BMT is currently used for the treatment of various disease states. The results we have obtained suggest that the use of activated NK cells may be of considerable use in improving the efficacy of this procedure through the abrogation of graft rejection by the recipient and accelerating immune reconstitution. These results also suggest that NK cells may play a critical role in the maintenance of normal hematopoiesis, both as up and down regulators, depending on the circumstances. Additionally, these NK cells may provide a potent antitumor effect when BMT is used to treat cancer. We have also found that the neuroendocrine system exerts powerful effects on immune development and function. GH may be of considerable use as a means to also accelerate immune reconstitution and may be of use to treat immune deficiency states such as AIDS or in BMT.

SUMMARY REPORT

BIOLOGICAL RESOURCES BRANCH

October 1, 1990 through September 30, 1991

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 the 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

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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 (BOC), the 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. 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 (BOC)

The BOC acts as the steering committee for the BRMP extramural program. The BOC reviews and prioritizes agents as to evaluation in the preclinical screens. The recommendation of the BOC are subsequently presented to the DNC and in turn the recommendations of the DNC are implemented by the BOC and program staff. Members of the BOC 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 91 ACTIVITIES

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

* Maintained 24 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 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, IL-8, tumor necrosis factor (TNF)-alpha, TNF-beta, granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and transforming growth factor (TGF)-alpha, TGF-beta.

* Initiated plans to construct a pilot plant for the production of clinical-grade monoclonal antibodies and recombinant proteins at the Frederick Cancer Research and Development Center.

* Issued a Cooperative Agreement for clinical trials with BRMs.

* During FY 91, the BRB awarded $5.4 million for extramural contracts, including $2.7 million in clinical contracts and $2.7 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 91

A total of 107 grants were supported by the BRMP with $28.9 million during the period October 1, 1990 through June 5, 1991. There were 77 Research Projects (R01), 8 Program Project (P01), 1 Outstanding Investigator Grant (R35), 4 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. One Program Project is studying the use of anti-CD19 and CD22 monoclonal antibodies that have been conjugated to a deglycosylated ricin A chain. These immunotoxins will be used to treat B-cell lymphoma in Phase I/II clinical studies.

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 with 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 91 included studies focused on improving the heterogeneous distribution of antibodies, the development of immunotoxins for the treatment of Hodgkin's disease, the role of the antigen receptor gene in T-cell malignancies, and immunotherapy using ras-specific T-cells.

Request for Applications

In FY 91, 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), "Immunologic Investigation of Multi-Drug Resistance" was awarded.

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. Applications continue to be received in FY 91.

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. Two Task Orders were competed during this fiscal year, and Synthecell Corporation of Rockville, Maryland was selected to perform these Master Agreement Orders. One Task Order requires the synthesis of variable length (10-21 mers) phosphodiesters to inhibit the expression of Epstein-Barr virus proteins and the c-myc oncogene in Burkitt's tumors. A second Task Order was awarded to synthesize sense and antisense 15-mer oligonucleotides to study the role of IL-6 on the growth of myeloma and neuroepithelioma cells. Both Task Orders will be used to synthesize larger amounts of interesting sequences for in vivo studies.

A new RFP entitled: "Preparation of Monoclonal Antibody Chelates for use in Radioimmunotherapy" utilizes a Master Agreement mechanism which will be awarded in late FY 91. A specific task order has been included in this RFP for the chelation of the monoclonal antibody, Lym-2, under Good Manufacturing Practices conditions.

Three Task A contracts for Phase I clinical studies of monoclonal antibodies and immunoconjugates were maintained with the University of Alabama, the University of Texas, M.D. Anderson Cancer Center, and Memorial Sloan-Kettering Cancer Center, and four Task B contracts were maintained for Phase I clinical studies of cytokines at Cleveland Clinic, University of California-Los Angeles, University of Wisconsin, and Memorial Sloan-Kettering Cancer Center (Table III).

The BRB maintained 24 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. More than 200 grams of material have been delivered to the BRB.

In FY 91, the BRB received 110 grams of a total of 210 grams of monoclonal antibody R24 from Celltech Limited, which is a subcontract of Program Resources, Incorporated. A contract through Program Resources, Inc./DynCorp was finalized with Brunswick Biotechnetics for the production of clinical-grade anti-GD2 monoclonal antibodies and was completed with approximately 60 grams of material produced.

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 91 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.

CONFERENCES AND MEETINGS

* On September 12-13, 1991, the Biological Resources Branch will co-sponsor (with the Cancer Therapy Evaluation Program) an international conference on the use of IL-2 in hematologic malignancies. The conference will be held in Bethesda, Maryland. Proceedings will be prepared for publication.

* 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 31 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, Rousell-UCLAF, 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.

A Biological Imaging Group (BIG) was formed to discuss development of promising imaging/anti-metastatic agents. The pentapeptide, YIGSR, which is part of the B1 chain of laminin has been the focus of several meetings between members of the BRMP, CTEP, RRP, and Dr. H. Kleinman (NIDR). Preliminary data provided by Dr. Kleinman's laboratory on YIGSR has been used by Cytogen Corporation to design modified peptides that can chelate ^{99m}Tc for biodistribution and imaging studies. Cytogen Corporation is interested in working with Dr. Kleinman and the NCI to develop this compound as an imaging agent for metastatic disease.

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 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).

* An RFA for "Cooperative Agreements for the Study of Cancer Therapy with Biological Response Modifiers (CABRMs)." Awards are planned in FY 92.

* Establishment of a new Master Agreement mechanism for the Large-Scale clinical-grade production of cancer-specific targeting agents. These Master Agreements will give greater flexibility to the BRB's efforts to produce clinical-grade cancer targeting molecules, and will replace the single technology contract now in its final year.

PUBLICATIONS

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TABLE I

BIOLOGICAL RESPONSE MODIFIERS PROGRAM

BIOLOGICAL RESOURCES BRANCH

GRANT EXPENDITURES FOR FY 91 THROUGH JUNE 1991

TYPE OF GRANT	NUMBER	TOTAL COST AWARDED
Research Projects (R01)	77	$14,372,342*
Program Projects (P01)	8	10,416,886
Small Business Innovative Research (SBIR)	6	481,369
Conference Grants (R13)	1	15,000
Merit Awards (R37)	8	2,231,299
First Awards (R29)	6	547,376
Outstanding Investigator Award (R35)	1	685,586
TOTALS	107	$28,749,858*

*Included in this category are 4 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 Hodgkin's Disease"
CA52298	Kaminski, M. S.	"Anti-Tumor Effects of Anti-Idiotype Antibodies"
CA54168	Thorpe, P. E.	"Immunotoxins for the Treatment of Hodgkin's 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"

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)		
CA47829	Denardo, G. L.	"Studies to Improve Cancer Therapy with Antibodies"
CA33399	Levy, R.	"Anti-Idiotype Therapy of Human B-Cell Malignancy"
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		
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"

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)		
CA42396	Irie, R. F.	"Treatment of Melanoma With Human Monoclonal Antibody"
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 FCRIII Monoclonal Antibodies"
CA49856	Raso, V. A.	"Targeting Toxins with Acid-Triggered Hybrid Antibodies"
CA52304	Bignami, G. S.	"Bi-Specific Antibody for Directed Delivery of Polytoxin"
Monoclonal Antibody Localization		
CA43544	Murray, J. L.	"Optimization of Monoclonal Antibody Localization"
CA49792	Jain, R. K.	"Heterogeneous Distribution of Antibodies in Tumors"
Multi-Drug Resistance - Preclinical		
CA48146	Hochman, J. D.	"Monoclonal Antibodies to Human Multi Drug Resistance"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
CYCLOPHOSPHAMIDE		
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity By Cyclophosphamide"
Preclinical		
CA30088	Dray, S.	"Synergy of Tumor Chemotherapy and Host Immunity"
MOLECULAR BIOLOGY		
Preclinical		
CA48162	Croop, J. M.	"Immunologic Analysis...Multidrug Resistant Gene Family"
CA52539	Theofilopoulos, A. N.	"Antigen Receptor Genes in T-Cell Malignancies"
CA41746	Murphy, J. R.	"Diphtheria Toxin-Related Peptide Hormone Gene Fusions"
CA52527	Ostrand-Rosenberg, S.	"Enhancing Tumor Immunity by Class II Gene Transfection"
AIDS		
CA43447	Bolognesi, D. P.	"Preclinical Studies of Prevention and Intervention in AIDS"
LIPOSOMES		
Preclinical		
CA24553	Huang, L.	"Targeting of Liposome to Tumor Cells"
CA37528	Lee, V	"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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LIPOSOMES - Preclinical (Continued)		
CA39448	Matthay, K. K.	"Treatment of Leukemia by Antibody-Directed Liposomes"
CA25526	Papahadjopoulos, P. D.	"Liposome Targeting to Tumor Cells In Vivo"
CA53517	Anderson, P. M.	"Il-2 Liposomes in Cancer of the Lung"
TUMOR VACCINES		
Preclinical		
CA29592	Kahan, B. D.	"Active Specific Immunotherapy in Man: A Murine Model"
CA51434	Kohler, H.	"Preclinical Anti-Idiotype Therapy of Cancer"
CA49675	Ravdin, P. M.	"Autocrine Growth Factors--Effect of Immunoneutralization"
Clinical		
CA39248	Berd, D.	"Augmentation of Human Immunity by Cyclophosphamide"
CA36233	Mitchell, M. S.	"Specific Active Immunotherapy of Human Melanoma"
CA40532	Livingston, P. O.	"Immunization of Melanoma Patients with Gangliosides"
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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
LYMPHOKINES OTHER THAN IL-2		
Interferons - Preclinical (Continued)		
CA43201	Ozer, H.	"Immunoregulatory Effects of the Interferons"
Interleukin-1 - Preclinical		
CA38043	Lachman, L. B.	"Biologic Studies of Human Interleukin-1"
CA45143	Gaffney, E. V.	"Malignant Cell Growth Modulation by 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"
Tumor Necrosis Factor - Clinical		
CA49987	Epstein, A. L.	"Tumor Necrosis Treatment of Human Cancer"
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"
Other Lymphokines - Preclinical		
CA45672	Lilly, M.	"Therapeutic Uses for Human Granulocyte CSF"
CA52592	Yee, D.	"Growth Regulation of Cancer By IGF-I"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
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"
Clinical		
CA43950	Mier, J. W.	"Immunobiology of LAK Cells"
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"
CA54561	Cheever, M. A.	"RAS-Specific T-Cell Mediated Immunotherapy"
CA39286	Mastrangelo, M. J.	"Developing T-cell Clones Cytoxic to Sarcomas"
CA48109	Krueger, G.	"Suppressor T-Cell Manipulation as a Cancer Immunotherapy"
CA49231	Shu, S.	"Adoptive T-cell Immunotherapy of Nonimmunogenic Tumors"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
AUTOLOGOUS SPECIFIC ANTI-TUMOR CYTOXIC CELLS		
Clinical		
CA41741	Braun, D. P.	"Arachidonic Metabolism in Cancer Patient Macrophages"
CA51220	Chang, A. E.	"Adoptive Therapy of Human Cancer With Sensitized T-Cells"
CA52499	Belldegrun, A. S.	"Tumor Specific TIL From Renal Cancer"
TUMOR ANTIGENS		
Preclinical		
CA33049	Oettgen, H. F.	"Monoclonal Antibodies in the Treatment of Cancer"
CA42508	Reisfeld, R. A.	"Molecular Profile of Melanoma and Neuroblastoma Antigens"
INVESTIGATIONS OF MATURATION OR DIFFERENTIATION INDUCERS		
Preclinical		
CA42006	Choi, Y. S.	"Human B-cell Growth Factors"
CA17393	Donahoe, P. K.	"Muellerian Inhibiting Substance"
CA47589	Santoli, D.	"Growth Factor-Dependent Normal and Leukemic Cell Growth"
CA48080	Young, R.	"Myeloid Differentiation Therapy Reduces Suppressor Cells"
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"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
BONE MARROW TRANSPLANTATION		
Preclinical (Continued)		
CA18221	Storb, R. F.	"Marrow Grafting for Treatment of Hematologic Malignancies"
CA36725	Vallera, D. A.	"Immunotoxins in Human Bone Marrow Transplantation"
METASTASIS		
Preclinical		
CA30276	Niederkorn, J. Y.	"Immunologic Modulation of Ocular Tumor Metastasis"
CA49950	Barna, B. P.	"Synthetic CRP Peptide--Therapy for Cancer Metastases"
CA41525	Frost, P.	"Alien Gene Transfection in the Therapy of Metastasis"
TOXINS		
Preclinical		
CA42354	Maxwell, I. H.	"Tumor Cell Killing by Expression of a Toxin Gene"
CA41746	Murphy, J. R.	"Diphtheria Toxin Related Peptide Hormone Gene Fusions"
SBIR		
Phase I		
CA51568	Raychaudhuri	"Anti-T Cell Receptor Antibodies for Cancer Immunotherapy"
CA52323	Huston, J. S.	"Humanized Antitumor Binding Sites: Framework Engineering"
CA53938	Chiang, Y. L.	"Retroviral Vectors to Express Alpha-IFN in Human TIL"
CA52304	Bignami, G. S.	"Bispecific Antibody for Directed Delivery of Palytoxin"

TABLE II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
SBIR		
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)"
CA43969	Williams, R. M.	"Quinocarcin Mechanism of Action"
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		
CA55284	Royston, I.	"6th 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 II (CONTINUED)

GRANTS LISTED BY TOPIC AREA

TOPIC GRANT NUMBER	PRINCIPAL INVESTIGATOR	GRANT TITLE
DRUG DELIVERY		
Endocytosis		
CA14551	Ryser, H. J.	"Penetration of Macromolecules into Mammalian Cells"
DRUG RESISTANCE		
CA37082	Israel, M.	"Preparation of Adriamycin Analogs and Derivatives"

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* IL-2 plus TIL
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-melanoma Monoclonal Antibody and rIL-2
University of Alabama at Birmingham	Anti-melanoma Monoclonal Antibody
University of Texas M.D. Anderson Can. Ctr.	Anti-melanoma Monoclonal Antibody
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*
	IL-1 plus IL-2
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
	Anti-melanoma Antibody
Thomas Jefferson University	Anti-melanoma Antibody/Delayed Hypersensitivity
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*
	GM-CSF and Anti-Melanoma Antibody

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	
Collection, Storage, Distribution, and Quality Assurance of BRM	527
Animal Lymphokine Evaluation	230
Preclinical Evaluation Laboratory	670
Animal Production	350
Specialized Antibody Production	80
Monoclonal Antibody Purchases	27
Oligonucleotides	98
Monoclonal Antibody Chelates	220
Clinical	
Master Agreement Orders	322
Phase I Contracts	
Task A: Phase I Clinical Trials of Monoclonal Antibodies	1,101
Task B: Phase I Clinical Trials of Cytokines	375
Total	$4,000

SUMMARY REPORT

LABORATORY OF MOLECULAR IMMUNOREGULATION

October 1, 1990 through September 30, 1991

INTRODUCTION

Headed by Dr. Joost J. Oppenheim, the Laboratory of Molecular Immunoregulation (LMI) performs basic research studies on host defense mechanisms. The role of cytokines and immune responses in host defense are the primary focus of the LMI. Cytokines 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: Immunobiology, Lymphokine, and Cytokine Molecular Mechanisms. In addition, a fourth research group in the LMI concentrates on studies of inflammatory cytokines and immune mechanisms, 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 David Kelvin are investigating cytokines involved in hematopoiesis, carcinogenesis and inflammation, Dr. Scott Durum is studying cytokines involved in immunity and Dr. Louis Matis is investigating the contributions of T lymphocyte mediated processes to host defense.

Dr. Oppenheim, the laboratory chief, previously observed that IL 1 is radioprotective in mice, and that the effect was due to restoration of hematopoiesis. These studies were performed in collaboration with Dr. Ruth Neta of the Armed Forces Radiobiology Research Institute (AFRRI).

Pre-administration of cytokines, in particular of 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 radioprotective effects of the cytokines were based on the recovery of suppressed hematopoietic tissues. Administration of inhibitory anti-IL 1 receptor and anti-TNF antibodies both reduced the radioresistance of normal mice and completely blocked the radioprotective effect of endotoxin. We determined that transforming growth factor ß (TGFß) also radiosensitized mice, perhaps in part by reducing IL 1 receptor expression on bone marrow and other cell types. Both IL 1 and TNF are potent inducers of mitochondrial MnSOD. Cells transfected with the cDNA of this intracellular radical scavenger became more radioresistant, and also more resistant to agents that generate oxygen radicals such as adriamycin, mitomycin C as well as to TNF and IL 1 itself. Conversely cells transfected with antisense cDNA for MnSOD became more sensitive. Thus induction of MnSOD may contribute to the radioprotective effects of cytokines.

Dr. Oppenheim and colleagues have 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 Dr. Nancy Colburn (CBS, LVC, NCI). They have used JB6, a non-tumorigenic 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. Of the cytokines e.g. IFNα, IL 6, IL 1 and TGFß with differentive activities that were tested, only TGFß synergized with retinoic acid in a dose-dependent manner in reducing the number of JB6 colonies induced by PMA. Conversely, TNFα had the opposite effect and like PMA promoted colony formation by both JB6 and 3T3 cells. Thus, both TGFß and TNF may have a role in carcinogenesis.

Dr. David Kelvin, a recently recruited Senior Staff Fellow, is actively continuing previous studies by Dr. Kouji Matsushima (Cancer Research Institute, Kanazawa University, Ishikawa, Japan) of the newly discovered family of chemotactic proinflammatory cytokines and their receptors. The members of the chemotactic intercrine family, namely MCAF and IL 8 that were previously cloned by our laboratory are transcriptionally and post-transcriptionally regulated. Modulation of expression of these proteins can be stimulated by IL 1, TNF, and PMA. Other immunomodulators such as dexamethasone can inhibit expression. Three dimensional analysis of these proteins using NMR and crystalography indicate that the amino terminal portion as well as the region around His 34 may be important for binding of IL 8 to its receptor. Dr. Kelvin and colleagues have observed in competition studies with various ligands from the IL 8 family that several ligands will bind to the IL 8 receptor, though with different affinities. Distinct species of IL 8 receptors have been identified on cross-linking gels. The MCAF receptor is also capable of binding other ligands from the same class of intercrines with different affinities. A soluble receptor assay has been developed and employed to permit the biochemical study and purification of the MCAF receptor. The receptor is a glycoprotein with a molecular weight of approximately 38-40k. cDNA cloning of both the IL 8 and MCAF receptor is in progress.

Dr. Scott Durum, a tenured senior investigator, has been studying the role of cytokines such as IL 1 in immunity. He and his colleagues have been studying how IL 1 serves as a cofactor, together with antigen, in T-cell activation. Studies of how the IL 1 controls gene transcription revealed that many of IL 1's effects on a variety of cell types are mediated by the transcription factor AP-1. IL 1 activates AP-1 in several ways: 1) first, preformed AP-1 proteins are activated, 2) these induce the promoter for the AP-1 component, c-jun and 3) the mRNA for c-jun is stablized. These actions result in production of high levels of AP-1 factors, which subsequently trigger the promoters of other genes, such as IL 1. Dr. Durum and coworkers are also investigating the mechanism of action of IL 1 using homologous recombination to eliminate the IL 1ß gene.

Lymphocytes have the unique ability to rearrange genes that are used for immune recognition. Little is understood of the rearrangement mechanism because it has not been possible to observe this process in vitro. Dr. Durum and colleagues have developed an in vitro organ culture in which extensive rearragement of the Vß genes occurs in 24-48 hr. This enables them to study the "signals" that initiate rearrangement of the genes for the T-cell receptor.

T lymphocytes are critical to survival, yet little is known of how the body controls their production. Dr. Durum and his coworkers have taken various approaches to try to accelerate T-cell production. IL 1 has the ability to stimulate pre-T cells to proliferate in vitro and develop the CD4 marker, but was not effective in vivo. It has been established that growth hormone promotes the expansion of post-thymic T cells in vivo, and this hormone will be evaluated in a clinical trial in AIDS patients.

Dr. Louis Matis, a newly tenured senior investigator in the LMI, Office of the Chief, has pursued studies of the mechanism of T-cell activation, tolerance induction and T-cell specificity and development. Structure/function analysis of T-cell receptor expression showed that regions of both αß and γδ antigen receptors corresponding to the complimentarity determining regions of the variable chains influence specificity and thus must play an important role in interaction with antigen/MHC ligands. Molecular analysis of endogenous murine superantigens was performed, with the goal of analyzing the expression of these superantigens in distinct cell type and their interaction with T-cell receptors. In addition, bacterial enterotoxin superantigens were shown to induce Vß-specific tolerance in peripheral T cells, both by clonal anergy and clonal deletion.

A general role for TCR/self antigen interactions in T-cell development was suggested by the direct demonstration of a requirement for positive selection in the functional maturation of thymocytes expressing the γδ receptor heterodimer. T cells expressing a class I MHC-specific γδ transgenic receptor that developed in a ß2 microglobulin (e.g. class I MHC) deficient transgenic mice, in response to receptor-mediated signals and did not exit from the thymus to populate peripheral lymphoid organs. This indicates that receptor-mediated signalling is a required step in the differentiation of this T-cell subset.

IMMUNOBIOLOGY SECTION

Dr. Luigi Varesio, acting head of the Immunobiology Section, studies how macrophages are activated (by various agents, including IL 2, IFNγ and endotoxin) to kill tumor cells. He and his colleagues have shown that IL 2 activates circulating monocytes via one subunit of the IL 2 receptor, the p75 subunit. Furthermore IL 2 augmented IL 2Rß without affecting IL 2Rα expression in monocytic cells. Induction of IL 6 and CSF-1 receptor was also associated with monocyte activation by IL 2. The discovery that IL 2 is a potent macrophage activating agent has profound impact on the potential therapeutic use of IL 2. Synergism between IL 2 and IFNγ or CSF-1 has been observed in the activation of cytotoxic activity. In contrast, inhibitory

effects on the activation by IL 2 are exerted by IL 4 and TGFß. The interaction between IL 2 and other cytokines is being analyzed at the levels of nuclear transactivating factors. The modulation by IFNγ of transactivating factors involved in promoting expression of IL 2Rα chain has been established. Finally, Dr. Varesio and coworkers have extensively analyzed the expression and the role of double stranded RNA-dependent enzymes in the activation of murine macrophages. The results are consistent with the hypothesis of a central role for these enzymes in the late phases of macrophage activation, and have led to the discovery of the unique macrophage activating properties of picolinic acid, a metabolite of tryptophan. These results provided the first evidence of a direct role of tryptophan metabolites in macrophage activities.

Dr. George Cox, a newly recruited senior staff fellow in the Immunobiology Section, has investigated the interrelationship of IFNγ and IL 2 in the activation of murine macrophage cell lines (Mϕ). The Mϕ cell line ANA-1 does not express detectable levels of mRNA for the p55 subunit of the IL 2 receptor (IL 2Rα) constitutively. However, ANA-1 Mϕ express low levels of the p70-75 subunit of the IL 2 receptor (IL 2Rß) on their cell surface, and treatment of these Mϕ with IFNγ induces the expression of IL 2Rα mRNA and protein. Mϕ cell lines and normal murine peritoneal Mϕ do not express constitutive cytotoxic activity against p815 mastocytoma cells in vitro. Moreover, these Mϕ, in contrast to normal human monocytes and rat Kupffer cells, do not become tumoricidal after treatment with IFNγ alone or IL 2 alone. However, Mϕ become activated after coincubation with IFNγ plus IL 2. Studies designed to identify inhibitors of Mϕ tumoricidal activity revealed that IL 4 inhibits IFNγ plus IL 2-induced, but not IFNγ plus LPS-induced cytotoxicity. At least one mechanism of IL 4 suppressive activity appears to be due to its ability to reduce the IFNγ-induced expression of IL 2Rα mRNA in Mϕ.

Surprisingly, Dr. Cox has observed that murine Mϕ cell lines activated by IFNγ express several genes that are homologous to murine IL 2. A molecular cloning approach was utilized to explore the expression of novel IFNγ-inducible genes in murine Mϕ. A cDNA library was prepared using mRNA isolated from an IFNγ-treated Mϕ cell line. Several cDNA clones were isolated from the library using a fragment of the murine IL 2 cDNA as a probe. Sequence analysis indicated that two of these clones represent novel genes. Additional studies indicated that IFNγ plus IL 2-treated Mϕ are induced to express several genes that encode chemotactic cytokines (e.g. JE, KC, and CSF-1).

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 TGFß. His group has shown that TGFß had several potent bifunctional effects on hematopoiesis. TGFß1 and TGF ß2 are equipotent selective inhibitors of hematopoiesis that halt the growth of early human and murine progenitors, but not more differentiated progenitors. Using partially purified murine hematopoietic stem cells in single cell assays with several cytokines, it was shown that TGFß acted

directly on progenitor cells to block growth. In contrast, addition of TGFß to GM-CSF stimulated bone marrow cells greatly augmented growth, leading predominantly to an increase in granulocytes, and was also shown to have a direct effect in single cell assays. Both in vivo and in vitro experiments with TGFß show that hematopoietic stem cells are reversibly prevented from entering the cell cycle. These growth modulatory effects function at, at least, two levels: 1) trans-down modulation of the cell surface receptors for positive regulatory signals and/or 2) interference with post-receptor signalling of these molecules by decreasing cytokine mediated phosphorylation or by inhibiting specific gene expression.

Dr. Ruscetti and colleagues have also made vital observations regarding roles of TGFß in neoplasia. Leukemic cell lines could be either sensitive or insensitive to TGFß mediated growth inhibition. Growth of neoplastic B lymphocytes can occur by escaping from a TGFß mediated autocrine inhibitory loop. Activation signals (e.g. phorbol esters) can inhibit tumor cell growth by stimulation of active TGFß production and induction of cell surface expression of functional TGFß receptors. In studying effector cell function by CD3+ and CD3- lymphocytes, it was found that cell mediated cytotoxicity was much more sensitive to TGFß mediated inhibition than cell growth and that expression of the IL 2 receptor p55 chain was inhibited by TGFß while expression of p75 IL 2 receptor chain was not; thus reducing the lymphocytic response to IL 2.

To better understand the effects of host cells on viral replication as well as the interaction between infected cells and the immune system, Dr. Ruscetti and his colleagues have developed in vitro models of viral latency and restricted viral expression for both HTLV-I and HIV-I. In HTLV-I infected B cells from acute T-cell leukemia (ATL) patients, very few 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 poorly expressed. Also, the macrophage, a reservoir of HIV-I infection in AIDS patients, was shown to be able to restrict HIV-I expression. THP-1, a macrophage cell line, was permissive for HIV-I expression. However, these infected cells become naturally non-productive for HIV 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 nucleus 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 these host 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, Dr. Ruscetti finds that negative regulation of chronic expression in monocytes is mediated through inhibition of binding of the transcription factor, NF-kB, to the HIV enhancer.

CYTOKINE MOLECULAR MECHANISMS SECTION

Dr. William Farrar, head of the Cytokine Molecular Mechanisms Section, and his colleagues have studied the molecular mechanisms of action of several cytokines, including IL 2, IL 3 and GM-CSF, and erythropoietin (Epo). 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 these receptors are tightly associated with one or more tyrosine kinases. They 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. In addition, they have examined the cytoplasmic domains of the Epo receptor through mutation and functional analysis and have deduced the regions required for growth promotion and protein kinase coupling. The laboratory has cloned five new human protein kinases which are expressed in normal and leukemic tissues. One tyrosine kinase designed RLK appears to be a member of a unique family of PTK recently discovered in 1990. This kinase has two catalytic domains and contains some domain similarities to the c-fms proto-oncogene. This gene is located on human chromosome 1 and IFN γ upregulates the mRNA expression of this gene in human monocytes. 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, suggesting that certain pharmacological inhibitors can block HIV transcription at this level.

Dr. Diana Linnekin, a newly recruited staff fellow in the Cytokine Molecular Mechanisms Section has investigated phosphorylation by kinases in cytokine activated cells. She has identified a highly conserved serine kinase substrate to be the actin binding protein 1-plastin. The factor-induced phosphotyrosylprotein has several characteristics consistent with those of a tyrosine kinase. Further, this tyrosine kinase can be found in association with the Epo receptor and is constitutively phosphorylated in cells rendered Epo-independent through either transformation with Friends spleen focus forming virus (SFFV) or transfected with gp55, the envelope glycoprotein of the SFFV. In addition, her work also suggests that this protein may be associated with other cytokine receptors and may represent a protein tyrosine kinase critical for signal transduction of a number of cytokines.

OVERVIEW OF LMI ACTIVITIES

The LMI studies the relationship of cytokines to the immune response, host defense processes and tumor cell growth and differentiation. In addition to pursuing their own aforementioned studies, LMI scientists are engaged in many outside activities.

In addition to contributing book chapters and reviews to many journals, LMI investigators are also invited to present their findings at many national and international meetings. LMI investigators also serve on peer review study sections, organize and teach post graduate courses, participate in site visits and program reviews, consult at non-NIH institutions and serve as editors of journals. Thus LMI scientists contribute their experience and points of view to the scientific community.

LMI scientists have been involved in the organization of meetings such as the International Cytokine and Lymphokine Workshops, the Leucocyte Biology meetings, the meetings of the American Association of Immunologists, the International Immunology Congress and more. These meetings promote communication and collaborative interactions among scientists and their proceedings are also communicated in books coedited by LMI investigators. Based on their scientific achievements and other activities the LMI scientific staff has been able to attract postdoctoral students and sabbatical scientists to work in their laboratories at the LMI, BRMP, NCI-FCRDC.

The staff of the LMI participate not only in weekly scientific meetings at the section and laboratory level, but also participate in BRMP and FCRDC retreats, seminars and meetings. There are several optional journal clubs and LMI scientists can subscribe to post-graduate training at the NIH or elsewhere as needed to promote their research progress. 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 the Clinical Research Branch (CRB), and a monthly meeting is held that brings together the clinical investigators with LMI and other basic scientists. This provides a forum that enables LMI investigators to contribute to formulating protocols for clinical evaluation of biological agents. 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.

There have been several noteworthy changes in the staff of the LMI over the past year. Dr. Louis Matis completed the transfer of his laboratory from the Bureau of Biologics of the FDA to the LMI by November 1990. He is now actively pursuing his research full time at the FCRDC. A number of collaborative studies have been initiated between Dr. Matis and LMI as well as BRMP staff which should be of mutual benefit. Dr. Kouji Matsushima resigned his position as a Visiting Scientist at the LMI in November 1990 to return to a position as Professor of Pharmacology at Kanazawa University. He will continue to visit twice yearly to assist us in completing our collaborative projects. His position has been taken by Dr. David Kelvin who is pursuing some of the chemotactic cytokine studies that are of programmatic interest to the LMI. Two additional staff fellows, namely Drs. George Cox and Diana Linnekin have joined the LMI and are strengthening the research programs of their respective sections. Although additional office space has been made available to BRMP scientists, the LMI is still short of laboratory space and scientists work space is crowded. Fortunately the budget for the LMI has been stable, but due to increased costs for laboratory supplies, equipment and salaries, this has actually resulted in some decrease in LMI operating funds.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09287-07 LMI

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.)*
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	Biologist	LMI, NCI
Other:	U. Csaikl	Visiting Fellow	LMI, NCI
	W. Murphy	IRTA	OAD, NCI
	D. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*
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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:
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 IL 1 controls gene transcription, and conclude that many of IL 1's effects on a variety of cell types are mediated by the transcription factor AP-1. IL 1 activates AP-1 in several ways: 1) first, performed AP-1 proteins are activated, 2) these induce the promoter for the AP-1 component, c-jun and 3) the mRNA for c-jun is stablized. These actions result in production of high levels of AP-1 factors, which sbsequently trigger the promoters of other genes, such as IL 2.

Lymphocytes have the unique ability to rearrange genes that are used for immune recognition. Little is understood of the rearrangement mechanism because it has not been possible to observe this process in vitro. We have developed an invitro organ culture in which extensive rearrangement of the Vß genes occurs in 24-48 hr.

T lymphocytes are critical to survival, yet little is known of how the body controls their production. We have taken various approaches to try to accelerate T cell production. IL 1 had the ability to stimulate pre-T cells to proliferate in vitro and develop the CD4 marker, but was not effective in vivo. We now find that growth hormone promotes expansion of post-thymic T cells in vivo, and this will be used in a clinical trial in AIDS patients.

PROJECT DESCRIPTION

PERSONNEL

Scott K. Durum	Biologist	OC, LMI, NCI
Ulrike Csaikl	Visiting Fellow	OC, LMI, NCI
William Murphy	IRTA	OAD, NCI
Dan L. Longo	Associate Director	OAD, NCI

OBJECTIVES

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

2) What is the physiological role of IL 1: Creating the IL 1-less mouse.

3) How do T cells rearrange the genes for their receptors?

4) What controls growth and development of T lymphocytes?

METHODS EMPLOYED

1. How Does IL 1 Control the Transcription of Genes?

To locate the IL 1-responsive elements in the c-jun and IL 2 promoters, constructs were used which contained wild type or deletion mutant promoters attached to a reporter gene, chloramphenicol acetyl transferase (CAT). These constructs were electroporated into HEPG2, a human hepatoma line, or LBRM331A5, a murine lymphoma line. IL 1 was used to trigger a response in these promoters, which was then measured by the amount of CAT enzymatic activity.

To measure the transcription of the jun and fos genes in response to IL 1, nuclear run-off analyses were performed.

To measure the levels of jun and fos mRNA in cells, Northern analysis was performed. To determine the effects of IL 1 on stability of these messages, HEPG2 cells were treated with phorbol ester, which induces increased jun and fos transcription, then treated with actinomycin D, which blocks further transcription, then IL 1. At various times thereafter, the level of jun and fos mRNA was determined and this reflected the effect of IL 1 on message stability.

To determine whether jun and fos were required in the response to IL 1, ATt20 pituitary cells were treated with anti-sense (or sense, as a control) oligonucleotides specific for the 5′ ends of jun or fos. Cells were stimulated with IL 1 and the effect on ß-endorphin secretion was measured.

2. What is the Physiological Role of IL 1? Creating the IL 1-Less Mouse

Our objective is to produce mice lacking the gene for IL 1ß, and this will be achieved using homologous recombination (the strategy is described in the Major Findings section below). We obtained murine IL 1ß genomic plasmid clones from John Telford (Sclavo, Italy), and murine IL 1α genomic cosmid clones from David Chaplin (Washington Univ., St. Louis, MO). Several IL 1ß targeting constructs were produced in our laboratory, by disrupting exon 7 with a promoterless neo gene. The D3 ES cell line is being grown in our lab, using as a feeder layer, embryo fibroblasts from mice with a neo transgene (this protects the feeders from massive death during selection with neo). ES cells will be electroporated with targeting constructs, and clones with targeted IL 1ß genes will be identified. Such clones will be introduced into blastulas, then allowed to develop in pseudopregnant foster mothers. Chimeric progeny will show patches of agouti coat (cells derived from the ES clone) against the black coat of the host. Male chimeric mice will be bred, and progeny tested for germline transmission of the targeted IL 1ß gene. Homozygous mice (with two targeted alleles) will be produced by mating heterozygotes. Extensive testing of these mice will be performed for abnormalities in development, immunity and inflammation.

3. How do T Cells Rearrange the Genes for Their Receptors?

Two methods are used to obtain cells that are rearranging their TcR genes. The first method is used to obtain small numbers of unrearranged pre-T cells, for purposes of observing stimuli for rearrangement. The thymus is removed from mouse embryos at d.14 of gestation. These cells are unrearranged, and if the whole organ is placed in culture for 24-48 hr. rearrangement occurs. Various antibodies or metabolic stimulants or inhibitors can be added to the culture and the effect on rearrangement is determined. The second method is used to obtain large numbers of pre-T cells that can be used to isolate the nuclear proteins that bind to the rearrangement sites. Mice are lethally irradiated, then recontituted with fetal liver cells. Eight to twelve days following irradiation, we find the rearrangement process is in full swing. These cells are used to prepare nuclear protein extracts, which are added to labeled double-stranded oligonucleotides containing the heptamer nonamer motif, and the effect on mobility in gels is determined. Poly-IC is added to this reaction to block non-specific binding.

The rearrangement process we observe is the joining of Vß8 to J. This is detected using a PCR reaction, in which primers are used corresponding to this V gene and J gene. If the two genes are unrearranged, they are too far apart for the PCR reaction to occur. If V and J are joined via the rearrangement process, we obtain a PCR product, whose specificity is verified by Southern hybridization with a probe for Vß8. Another test of specificity is through comparisons of mouse strains: the C57BL/6 mouse is generally used, whereas the SJL mouse lacks the Vß8 genes. This difference is also useful, for example in constructing fetal liver chimeras, C57BL/6 is used as donor, SJL as a recipient; thus, we know that all the rearranged cells derive from the donor.

4. What Controls Growth and Development of T Lymphocytes?

We used an in vitro culture to identify agents that stimulated the proliferation and differentiation of immature T cells. Double negative cells were purified from mouse and rat thymus using panning with anti-CD4 and anti-CD8 antibodies. Cells were cultured with various cytokines and proliferation was measured by ^{3}H-TdR incorporation, differentiation was measured by FACS analysis of various surface markers and mRNA expression for a number of genes.

For in vivo testing of various cytokines (IL 1, IL 2, and growth hormone [GH]), mice were injected IP with different doses (generally 5 μg) of the agent. The effect of these mediators on T cell expansion was determined in SCID mice which were injected with bone marrow or thymocytes from normal mice or humans. At various times thereafter, lymphoid organs were analyzed for T cell content by FACS.

MAJOR FINDINGS

1. How Does IL 1 Control the Transcription of Genes?

We previously examined the transcription factors induced by IL 1, in an effort to understand how IL 1 could elicit so many responses in so many different cell types. We determined that the transcription factor AP-1, which controls many genes, is induced by IL 1. AP-1 consists of a heterodimer of c-jun and c-fos proteins. Our recent studies focused on the mechanism whereby IL 1 activates AP-1, and we have found that this occurs at several levels. In the first ten minutes after IL 1 engages its receptor, preformed AP-1 protein becomes activated, thus triggering AP-1 sites on the c-jun promoter. This results in increased transcription of the jun gene. The jun mRNA that is produced is further stabilized in the presence of IL 1. The net effects of increased transcription and stabilization result in a twenty-fold rise in jun mRNA. The jun protein comprises half of the AP-1 transcription factor, the other half being the fos protein, whose mRNA is also increased by IL 1 in some cells. Consequently, by one hour after engaging the IL 1 receptor, the rise in AP-1 factors can induce many genes, including the IL 2 gene in T lymphocytes. We have extended our studies to two other important IL 1-responsive cell types, and similarly demonstrated a role for AP-1 in these responses: the hepatocyte, which is an important cell responding to IL 1 during the acute phase response in vivo, and the pituitary cell (in collaboration with
Drs. Fagarasan and Axelrod) which controls the response of the pituitary-adrenal axis to IL 1 in vivo.

2. What is the Physiological Role of IL 1? Creating the IL 1-Less Mouse

IL 1 has a multitude of effects, representing many cell types responding in a variety of ways. Most of these effects have been established by adding exogenous IL 1 to a system, either in vitro or in vivo. But which of these many responses are really important in homeostasis? We are approaching this question by deleting the IL 1 genes from a mouse. This involves a complex strategy, in which defective IL 1 genes will be used to replace the normal

gene by the process of homologous recombination in an embryonic stem (ES) cell line. Then these ES cells will be used to produce mice that contain the defective IL 1 gene. These IL 1-less mice will be extensively analyzed for physiological abnormalities.

We are in mid-course in this project. Thus far, we have produced several constructs with "defective" murine IL 1ß genes. These include exon 7 (the critical IL 1 exon) which has been rendered non-functional by disrupting it with a gene for neomycin resistance (neo). The strategy in designing the construct should allow us to distinguish between the rare homologous recombination events (in which the construct replaces the normal IL 1 gene) and the far more frequent random integration events. Thus we have used a neo gene lacking its own promoter; this neo gene will become functional only if the construct integrates into the genome, somewhere near a functional promoter, hopefully the IL 1ß promoter, meaning it has disrupted the normal IL 1 gene. Thus cells will be resistant to neomycin if they produce neo, but such cells will include both a) true homologous recombinants, as well as b) those in which the construct has integrated near some other promoter. To distinguish a) from b), we will perform polymerase chain reactions (PCR) and Southern analyses to determine which cell clones have true homologous recombinants.

Having produced the gene constructs, we are ready to begin the process of knocking out the gene in ES cells. We have experienced collaborators helping us in this phase, Brian Stanton and Luis Parada, who have successfully targeted several protooncogenes. We have successfully grown the D3 ES cell line in our laboratory.

3. How do T Cells Rearrange the Genes for Their Receptors?

Perhaps the most remarkable feature of the mammalian immune system is the gene rearrangement process. This rearrangement process is a controlled movement of several types of minigenes to distant areas on the same chromosome, followed by deletion of intervening DNA. Very little is understood of this process, primarily because it has not been possible to observe gene rearrangement in vitro, it has only been seen in intact animals. We are examining this gene rearrangement process in T cells, and are focusing on the TcRß gene, using a sensitive new PCR technique.

We find that the rearrangement of V to D begins in the embryonic thymus at day 15 of gestation. We have successfully produced an in vitro system where gene rearrangement occurs: the thymus organ is removed at day 14, placed in organ culture, and over the next 24-48 hr, extensive rearrangement takes place. We are now able to use this in vitro system to examine the nature of the signals that induce a pre-T cell to rearrange its genes. We find that the signal requires the architecture of the intact thymic organ, since neither crude supernatants nor a variety of defined cytokines could induce rearrangement of single suspensions of pre-T cells. We have no evidence yet for any cytokine being involved in this signal to rearrange, since antibodies to many cytokines

and their receptors (IL 1, IL 2, IL 3, IL 4, IL 10, TNF or INF γ) have had no blocking effect and adding exogenous cytokines to these organ cultures has not increased the rate of rearrangement.

We are now evaluating a number of surface structures on the pre-T cell (thy-1, PGP-1, J11, CD4, LY6, LFA-1, CD2, Ly1) and the epithelial cell (class I and II MHC, CD23) which might act as the signals to rearrange. In preliminary experiments, anti-class II MHC and anti-CD23 antibodies have strongly inhibited rearrangement, whereas anti-class I MHC antibodies have not inhibited. If these findings are substantiated, we will pursue the idea that class II and CD23 molecules on the thymic epithelium engage CD4 and an unknown CD23 receptor respectively on the pre-T cell, and these signals stimulate rearrangement.

We have also sought a system for studying the nuclear proteins that bind DNA during gene rearrangement. The preceeding functional studies used embryonic thymus, but this contains only a few thousand cells, so we looked for a larger source of pre-T cells that were undergoing rearrangement. We have successfully produced a large, synchronously rearranging population using radiation chimeras. Mice were irradiated and reconstituted with fetal liver (as a source of unrearranged pre-T cells). The thymus, 8-12 days following this reconstitution contains several million rearranging cells, and we know they derive from unrearranged cells from the donor, since the host lacks the particular Vβ gene that we examined. From these thymuses we have prepared nuclear extracts. We are currently using a band shift assay to detect proteins that bind the recombination site, and found a protein complex with apparent specificity for this site.

4. What Controls Growth and Development of T Lymphocytes?

The production of most hematopoietic cells is regulated by colony-stimulating factors, however, no analogous cytokine has yet been identified for lymphoid cells. It would be clinically useful to identify such a colony stimulating factor for T cells, which would enable us to stimulate the body to produce more T cells, for example in patients with AIDS and recipients with bone marrow transplants. We previously sought such an agent, as measured by induction of proliferation and differentiation of immature thymocytes in in vitro culture. We tested a variety of known cytokines and growth factors, and we also tested mixtures of uncharacterized cytokines using conditioned media from a number of different cells. We concluded that IL 1 had strong stimulatory properties on these immature T cells, inducing them to proliferate and differentiate into CD4 T cells. Thus, IL 1 appeared to have the properties we were looking for, but when we performed in vivo experiments, IL 1 failed to stimulate the thymus; instead, IL 1 administered in vivo induced a shrinking of the thymus, probably via induction of glucocorticoids and prostaglandins. We therefore turned to other candidates for the desired property of inducing T cell production.

We next tested growth hormone (GH) as a potential thymopoietic stimulant, an idea that was suggested by previous studies connecting pituitary activity with the size of the thymus. GH proved to have some activity in vivo in

stimulating thymopoiesis, but in the course of these studies, we found another effect of GH on T cells: GH powerfully induced expansion of post-thymic T cells, especially in the lymph nodes.

SIGNIFICANCE

1. How Does IL 1 Control the Transcription of Genes?

The demonstration that the AP-1 transcription factor mediates many of the nuclear effects of IL 1 helps explain why there are so many IL 1 effects, because many genes contain AP-1 motifs. Our studies are among the first to determine how cytokines induce transcription.

2. What is the Physiological Role of IL 1? Creating the IL 1-Less Mouse

It has not been previously possible to precisely determine the physiological roles of any gene, unless a natural mutant happened to be available. Using this new technology of homologous recombination, we will be able, for the first time, to mutate a specific gene in a whole mammalian organism, and thus pinpoint where and when a cytokine gene comes into play.

3. How do T Cells Rearrange the Genes for Their Receptors?

Gene rearrangement is the sine qua non of lymphocytes. The process has not been amenable to study, because there has been no in vitro systems for observing rearrangement. We have produced such a system, and should be able to use it to discover the signals which induce a pre-T cell to rearrange. The genetic machinery for rearrangement should also be amenable to study using several new approaches. Understanding the rearrangement process may reveal how undesirable gene rearrangements occur, resulting in cancer. A further benefit of understanding rearrangement is that we may be able to exploit this machinery to produce desirable changes in the genome, for example to introduce transgenes, and to perform homologous recombination.

4. What Controls Growth and Development of T Lymphocytes?

Although we have not yet determined what controls production of T cells, we have found a powerful effect of GH in stimulating expansion of peripheral T cells. This finding suggests application of GH in AIDS and bone marrow transplantation.

PROPOSED COURSE

1. How Does IL 1 Control the Transcription of Genes?

IL 1 induces mRNA for jun in some cells, and for both jun and fos in other cells. Thus some cells turn on the fos gene in response to IL 1, others do not. We would like to determine if the difference between cells is due to the difference in the two known IL 1 receptors, type I and type II. From the cell types examined thus far, it is possible that type I receptor activates only jun, whereas type II activates only fos (or possibly both jun and fos). We

will test this hypothesis in collaboration with John Sims (Immunex) using antibodies against the different receptors, as well as using cells transfected with expression vectors for the different receptors.

2. What is the Physiological Role of IL 1? Creating the IL1-Less Mouse

In the immediate future, we will continue the procedures towards obtaining mice that lack the IL 1ß gene. Once obtained, we will extensively analyze these mice for abnormalities in development, immunity and inflammation. These studies will be done both in our laboratory, as well as in many collaborating laboratories with specialized expertise. What if IL 1-less mice die in utero? Our collaborating embryologists, Stanton and Parada, would be very interested in pinpointing such an effect.

There is a second IL 1 gene, IL 1α, which we will next target. It is puzzling that there exist these two IL 1 genes, α and ß, since both are produced under similar circumstances, and seem to have the same actions. Yet despite this overlap, it seems likely that both α and ß genes are important, since they have been evolutionarily conserved in all mammals. We therefore predict that the IL 1α-less mouse will be different from the IL 1ß-less mouse. It will be difficult to produce a mouse lacking both IL 1α and IL 1ß, because the genes are linked. However, meiotic crossovers should occur between the loci, and we will try to obtain a double defective mutant through breeding and testing for such a crossover. If this fails, we can produce gene constructs with different selectable markers, enabling us to knockout both genes in the same ES cell, and this will be used to produce an IL 1α- IL 1ß-less mouse.

3. How do T Cells Rearrange the Genes for Their Receptors?

We will continue to search for the signals that induce a pre-T cell to rearrange its genes. Our studies will continue to test specific antibodies for blocking the signal to rearrange, and we will use other agents that can mimic the signals. The preliminary findings that an Ia-CD4 complex and a CD23-unknown receptor complex are involved will be pursued.

We will continue to seek proteins that bind to the relevant sites on DNA during rearrangement - if we verify that the protein we have already observed is indeed specifically binding the rearrangement site, we may be able to clone it by picking it out of an expression library made from rearranging T cells. We will try to exploit the recombinase system to introduce genetic constructs into T cells - thus we will attempt to introduce a foreign gene containing the rearrangement sequences.

4. What Controls Growth and Development of T Lymphocytes?

A clinical trial will be performed in AIDS patients, conducted by Dr. Robert Yarchoan (COP,NCI). GH and IGFI (the secondary mediatory of many GH effects) will be administered together with AZT (to arrest viral replication).

In the laboratory, we will continue to study the control of T cell production. Many basic phenomena have not been explained. Why does T cell production decline so dramatically with age (we will look for controls at the level of the bone marrow stem cell and at the thymic epithelial level)? Does the body have a way of knowing how many T cells there are, and thus a way of specifically regulating T cell production (we will specifically deplete T cells and observe the effect on thymopoiesis)? If a specific mechanism appears to control the number of T cells, we will evaluate previously identified cytokines for such an activity, and if none are successful, we will seek a novel cytokine.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09289-06 LMI

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.)*
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. W. 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); Biological Carcinogenesis and Development Program, PRI/DynCorp Frederick, MD (R. Hornung)

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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.)*

Pre-administration of cytokines, in particular of 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 radioprotective effects of the cytokines were based on the recovery of suppressed hematopoietic tissues. Administration of inhibitory anti-IL 1 receptor and anti-TNF antibodies both reduced the radioresistance of normal mice and completely blocked the radioprotective effect of endotoxin. We determined that transforming growth factor ß (TGFß) also radiosensitized mice, perhaps in part by reducing IL 1 receptor expression on bone marrow and other cell types. Both IL 1 and TNF are potent inducers of mitochondrial MnSOD. Cells transfected with the cDNA of this intracellular radical scavenger became more radioresistant, and also more resistant to agents that generate oxygen radicals such as adriamycin, mitomycin C as well as to TNF and IL 1 itself. Conversely cells transfected with antisense cDNA for MnSOD became more sensitive. Thus induction of MnSOD may contribute to the radioprotective effects of cytokines.

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. Of the cytokines e.g. IFNα, IL 6, IL 1 and TGFß with differentiative activities that were tested only TGFß synergized with retinoic acid in a dose-dependent manner in reducing the number of JB6 colonies induced by PMA. Conversely, TNFα had the opposite effect and like PMA promoted colony formation by both JB6 and 3T3 cells. Thus cytokines may influence the development and growth of tumorigenic 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 capacity of cytokines with growth and differentiation effects to counteract the action of immunosuppressive, bone marrow suppressive and carcinogenic agents are being evaluated. These activities of cytokines are being studied by investigating the in vivo pathways and mechanism of the radioprotective and reparative effects of cytokines such as Interleukin 1 (IL 1) and tumor necrosis factor (TNF) in mice. In addition, the potential role of differentiative cytokines in countering neoplastic transformation by tumor promoters such as phorbol myristic acetate (PMA) is being investigated in an in vitro model.

Radioprotective and Reparative Effects of Cytokines

In collaboration with Dr. Ruth Neta (AFRRI), we previously reported that prior administration of IL 1 and TNF, but not GM-CSF, G-CSF, IL 2 or IFNα,β have radioprotective effects and promote the survival of mice following 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. Thus, the administration of these two cytokines is more effective and appears to account for all the radioprotective effects of LPS. This hypothesis was challenged using antibodies to type I IL 1 receptor (anti-IL 1R) and to murine TNF that can block many of the effects of these cytokines. This approach revealed that both anti-IL 1R and anti-TNF decreased the resistance of normal mice to lethal radiation. The radiosensitizing effects of these antibodies suggests that they either interfere with normal low endogenous levels of IL 1 and TNF that contribute to normal radioresistance, or alternatively with the positive reparative feedback effects of IL 1 and TNF that are reported to be generated following radiation injury.

Adjuvant LPS Induces Radioprotective and Radiosensitizing Cytokines

Administration of anti-IL 1R (35F5 directed against type I IL 1R 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 net radiosensitizing effect. 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 radiosensitizing cytokine(s). Only a few of the many LPS induced cytokines are potential radiosensitizers. We reported previously that IL 6 by itself is radiosensitizing, but since it enhances the radioprotective effect of IL 1, IL 6 cannot account for these results. However, TGFß which has been shown to suppress the proliferation of early hematopoietic progenitor cells (see Annual Report of F. Ruscetti) is a likely candidate. In addition, LPS is known to stimulate macrophages to produce TGFß. Administration of TGFß to mice indeed lowered their resistance to lethal radiation. The mechanism of these radiosensitizing effects of TGFß has been studied in collaboration with F. Ruscetti et al. These studies have shown that TGFß markedly reduces the expression of receptors for IL 1 on a variety of myelomonocytic and lymphocytic cell lines as well as on normal murine bone marrow cells. This is the converse of the effect of IL 1 itself, as well as G-CSF, GM-CSF and IL 3 all of which upregulate IL 1 receptor expression on bone marrow cells (see Annual Report of F. Ruscetti). Thus synergistic interactions between IL 1 and CSFs at the receptor level also may contribute to the radioprotective effects of IL 1.

Role of MnSOD in Radioprotection

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) by IL 1 and TNF 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 and 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 still be radioprotected by IL 1. This indicates that induction of glucocorticoids by IL 1 and TNF does not account for radioprotection by these cytokines.

Possible Role of Cytokines in Tumor Promotion

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 begins to grow in an anchorage-independent fashion 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 recombinant cytokines such as IL 1, IFN, IL 6, TNF and TGFß was tested and only TGFß was active in counteracting the in vitro effect of PMA on JB6 cells. The possibility that TGFß mediates the effect of retinoic acid (RA) was investigated and RA did not induce TGFß production, but upregulated the expression of receptors for TGFß on JB6 cells. However, since anti TGFß 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 finding suggest that TGFß 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 both for JB6 epithelial and 3T3 cells.

PROPOSED COURSE

IL 1 has considerably greater toxic side effects in man than IL 3, IL 6, G-CSF and GM-CSF. Consequently combinations of these cytokines are being tested further with Dr. R. Hornung (PRI) to establish the combinations of IL 1 and G-CSF, IL 3, IL 6 and GM-CSF yielding maximal radioprotective effects. In addition, 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 will be more thoroughly explored. The capacity of IL 1 to interact with other radioprotective agents such as anti-asialo GM-1 and WR 2721, an oxygen radical scavengers, is being investigated with Dr. J. Ortaldo.

Although our in vitro studies of an epithelial cell line suggest that TGFß synergistically enhances the antipromoter activities of retinoic acid. The relevance of this observation needs to be experimentally ascertained using other cell lines and by studying the in vivo effect of TGFß on tumor promotion. The mechanistic basis for the antipromoter activity should be explored by evaluating the effect of TGFß on anti-oncogenes and on DNA repair enzymes. Furthermore, the possibility that other differentiative cytokines may have analogous antipromoter effects in other tissues should be explored by purifying and identifying such activities when present in crude cytokine-

containing supernatants. Finally, the possibility that some cytokines such as TNF may emulate promoter activities and may lead to identification of antogonists that can counter such effects.

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 and tumorigenicity 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 09358-01 LMI

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.)*
Development, Function and Specificity of T Lymphocytes

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

PI:	L. A. Matis	Senior Investigator	LMI, NCI
Others:	M. M. Cotterman	Microbiologist	LMI, NCI
	C. D. Jarvis	IRTA Fellow	LMI, NCI

COOPERATING UNITS *(if any)*
Department of Biology, University of California, San Diego, La Jolla, CA (S.M. Hedrick); Department of Pathology, University of Chicago, Chicago, IL (J.A. Bluestone); BCDP, PRI/DynCorp, Frederick, MD; (F.B. Wells, F. Macchiarini).

LAB/BRANCH
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NCI-FCRDC, Frederick, Maryland 21702-1201

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 development, activation, and specificity of T lymphocytes were investigated. Structure/function analysis of T cell receptor expression showed that regions of both αβ and γδ antigen receptors corresponding to the complimentarity determining regions of the variable chains influence specificity and thus must play an important role in interaction with antigen/MHC ligands. Molecular analysis of endogenous murine superantigens was performed, with the goal of analyzing their expression in distinct cell type and their interaction with T cell receptors. Bacterial enterotoxin superantigens were shown to induce Vβ-specific tolerance in peripheral T cells, both by clonal anergy and clonal deletion.

A general role for TCR/self antigen interactions in T cell development was suggested by the direct demonstration of a requirement for positive selection in the functional maturation of thymocytes expressing the γδ receptor heterodimer. T cells expressing a class I MHC-specific γδ transgenic receptor did not proliferate to receptor-mediated signals and did not exit from the thymus to populate peripheral lymphoid organs when they developed in a β2 microglobulin (e.g. class I MHC) deficient environment. This indicates that receptor-mediated signalling is a required step in the differentiation of this T cell subset.

PROJECT DESCRIPTION

PERSONNEL

Louis A. Matis	Senior Investigator	OC, LMI, NCI
Melissa M. Cotterman	Microbiologist	OC, LMI, NCI
Christopher D. Jarvis	IRTA Fellow	OC, LMI, NCI

OBJECTIVES

To understand the basic mechanisms underlying T lymphocyte differentiation, growth, and in vivo function.

MAJOR FINDINGS

We have engaged in a series of studies to examine the differentiation, activation, and molecular basis of antigen specificity of T lymphocytes. The aim of this work is to better understand the fundamental mechanisms that govern intrathymic development, transmembrane signalling, and ligand recognition by T cells expressing αß and γδ antigen receptor heterodimers. In addition to providing basic insights into the developmental biology and growth control of this important cell subset, it is hoped that this work will lead to more effective approaches to modulating T cell function in vivo in the treatment of autoimmune conditions and cancer.

1. Molecular Basis of T Cell Specificity.

We have continued to examine the regions of the T cell antigen receptor that are important for ligand recognition. Our results have demonstrated, both for αß and γδ receptors, that amino acid residues encoded within the putative complementarity determining regions (CDRs) of the variable chains of T cell receptors are critical in determining specificity for antigens and MHC molecules. Structural models of the TCR based on key sequence homologies with immunoglobulin (Ig) molecules have suggested that the TCR will have a conformation similar to that of an Ig Fab fragment. Sequences within the predicted third CDR, encoded by the V(D)J juntions, would contact antigen peptides bound within the pocket of the MHC molecule, and sequences within the CDRs 1 and 2 should directly bind to MHC. Our results have provided evidence supporting this model.

We established two class II MHC-specific TCR γδ T cell clones, one specific for the I-A^d antigen, the other recognizing I-$E^{k,b}$ molecules, and sequenced their TCR genes. We found that both T cell clones expressed identical VδDδJδ and VγJγ genes, differing in sequence only in junctionally encoded residues resulting from inexact V(D)J joining and N region nucleotide addition. This result established the critical role of CDR3 residues in antigen recognition by the TCR γδ and indicated that there are likely to be fundamental similarities in the molecular basis of the TCR-ligand interactions of TCR γδ

and αß T cells. Presumably the conformational structures of both classes of TCRs are closely related.

We also determined that a single amino acid residue substitution in the CDR1 of the ß chain of an αß TCR could profoundly alter TCR usage in an antigen-specific MHC-restricted immune response to the model peptide antigen pigeon cytochrome c(cyt). Previous work has shown that most class II MHC I-E^k restricted cyt-specific T cells in B10 background mice express Vß3/Vα11 heterodimers. T cells from C57BR mice with very similar antigen and MHC specificity express Vß3 and Vα11, but almost never co-express these two chains. Detailed sequence analysis revealed that the differential receptor usage could be attributed to a single amino acid substitution of val to phe at residue 31 of the Vß3 protein, which lies within the predicted CDR1. This result confirmed the importance of Vß CDR1 in TCR antigen recognition, and indicated further that naturally occuring allelic polymorphisms in TCR V regions could represent an important source of diversity in the TCR repertoire. Differential TCR usage in antigen-specific responses resulting from allelic polymorphisms could explain associations between TCR locus haplotypes and disease susceptibility.

2. Role of Superantigens in the Immune Response Induction of Immunological Tolerance.

The term superantigen defines a class of molecules that activates all T cells expressing particular Vß proteins, regardless of the other components of the TCR, and thus that has the capacity to stimulate a high percentage of peripheral T cells compared to conventional peptide antigens. The bacterial toxins and the murine Mls antigens, the latter now shown to be proteins encoded by an open reading frame within the LTR of mouse mammary tumor retroviruses, are among the antigens with these properties. The superantigens have been shown to exert profound effects in vivo upon T cell development and function.

We had previously shown, along with others, that MlS antigens induce tolerance by clonal deletion within the thymus, of developing T cells expressing the Mls-reactive TCR Vß proteins. The Mls proteins are encoded by a 1.7 kb mRNA assembled by the splicing of a 5′ leader sequence encoded within the upstream LTR to a 3′ acceptor sequence just upstream of the downstream LTR and the ATG codon of the LTR ORF. We have examined the expression of this mRNA in B cells and T cell subsets using PCR technology, and have cloned and sequenced PCR generated products to explore the diversity of distinct Mls-like molecules expressed by antigen-presenting cells. In addition, we have cloned the MMTV ORF genes into expression vectors in order to study the function of these proteins by in vitro transfection analysis and in vivo through the construction of transgenic mice. Thus far this work has shown that the naturally spliced LTR ORF mRNAs that are presumably translated into endogenous superantigen proteins are indeed expressed in LPS-stimulated ß cells as well as ConA activated CD8+ and to a lesser extent CD4+ T cells. DNA sequencing has revealed significant diversity, indicating that at least several distinct MMTV ORF products are expressed within the lymphocytes of an individual mouse strain.

Our lab as well as others have also found that in vivo administration of superantigens can induce tolerance in peripheral T cells expressing appropriate specific Vß proteins. For example, parenteral injection of staphylococcal enterotoxin B(SEB) initially elicited a brief blastogenic response among Vß8+ murine T cells. However, the Vß8+ SEB-specific T cells were subsequently rendered unresponsive to further stimulation with SEB and ultimately were subject to programmed cell death. These experiments showed that peripheral T cells can be tolerized both by the induction of clonal anergy and by peripheral clonal deletion. Further analysis has shown that this form of peripheral tolerance to superantigens requires presentation by Ia molecules on APCs, and predominantly affects CD4+ T cells. Thus, it is possible to modulate in vivo the function of specific T cell subsets.

3. Positive Selection - The Role of TCR-Mediated Signals in T Cell Differentiation.

Studies of αß T cell development have shown that TCR/self antigen interactions in the thymus are critical for phenotypic and functional maturation of CD4+ CD8+ to CD4+CD8- and CD4-CD8+ thymocytes. This process is termed positive selection. We have shown that positive selection operates in the selection of TCR γδ-bearing $CD4^-CD8^-$ thymocytes, thus suggesting that TCR/self antigen interactions may represent a requisite step in all T cell development. This receptor-mediated signalling may be a universal property of all T cell differentiation.

To demonstrate positive selection of TCR γδ T cells, we bred mice expressing a class I-MHC specific γδ transgene to mice not expressing any class I MHC due to disruption of their ß2 microglobulin gene by homologous recombination. By analogy to TCR αß development, we reasoned that a γδ TCR alloreactive for a ß2M-associated class I MHC molecule would be positively selected during intrathymic development by a homologous syngeneic B2M-associated molecule. If so, the maturation of class I MHC-specific TCR γδ T cells might be impaired in a class I MHC deficient environment, just as mature CD8+ TCRαß+ T cells fail to differentiate in animals not expressing class I MHC. We showed that class I MHC specific TCR γδ transgenic T cells that developed in the thymus of mice not expressing ß2M-associated class I MHC molecules did not proliferate in response to TCR-mediated signals, and did not exit from the thymus and populate peripheral lymphoid organs. These results constituted direct functional evidence for positive selection of thymocytes expressing TCR γδ.

PROPOSED COURSE

Studies of TCR specificity will continue to examine the molecular basis of antigen recognition. Site directed mutagenesis and transfection analysis of T cell receptor genes will allow us to probe in great detail the critical functional regions of the TCR. Construction of transgenic mice expressing mutated TCR transgenes will similarly make possible the analysis of fine specificity of positive selection in the thymus.

Studies of T cell development and tolerance will be pursued by molecular and biochemical analysis of immature T cells that have failed to undergo positive

selection and of peripheral T cells that have been tolerized and have been programmed for cell death. We will examine second messenger expression and the function of kinases that appear to play a critical role in T cell signalling such as fyn, yes, and lck. Differential gene expression will also be examined through subtractive hybridization between positively selected and non-positively selected T cells and activated versus tolerized T cells. It is anticipated that these studies will provide insights into basic mechanisms of lymphocyte growth control.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09369-01 LMI

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.)*

Receptor Interactions and Signal Transduction by Inflammatory Cytokines

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

PI:	D. J. Kelvin	Senior Staff Fellow	LMI, NCI
Others:	J. J. Oppenheim	Chief	LMI, NCI
	A. Hishinuma	Special Volunteer	LMI, NCI
	J-M. Wang	Special Volunteer	LMI, NCI
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	J. A. Johnston	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

Department of Defense (A.O. Anderson); National Cancer Institute (E. Appella); Cancer Research Institute, Kanizawa University, Japan (K. Matsushima).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	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 members of the chemotactic intercrine family, namely MCAF and IL 8 are transcriptionally and post transcriptionally regulated. Modulation of expression of these proteins can be stimulated by IL 1, TNF, PMA and PKF. Other immunomodulators such as dexamethasone can inhibit expression. Three dimensional analysis of these proteins using NMR and crystallography indicate that the amino terminal portion as well as the region around His 34 may be important for binding of IL 8 to its receptor. Competition studies with various ligands from the IL 8 family show that several ligands will bind to the IL 8 receptor though with different affinities. Present studies are underway to identify the binding regions and the nature of ligands responsible for the low affinity binding. Distinct species of IL 8 receptors have been identified on cross-linking gels. These receptors presumeably have a different primary amino acid sequence and different affinities for IL 7. The MCAF receptor is also capable of binding other ligands from the same class of intercrines with different affinities. A soluble receptor assay has been developed and employed to permit the biochemical study and purification of the MCAF receptor. The receptor is a glycoprotein with a molecular weight of approximately 38-40k. cDNA cloning of both the IL 8 and MCAF receptor is in progress.

PROJECT DESCRIPTION

PERSONNEL

David J. Kelvin	Senior Staff Fellow	OC, LMI, NCI
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Atsushi Hishinuma	Special Volunteer	OC, LMI, NCI
Ji Ming Wang	Special Volunteer	OC, LMI, NCI
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Dennis F. Michiel	Visiting Fellow	OC, LMI, NCI
James A. Johnston	Visiting Fellow	OC, LMI, NCI

The staff of the laboratory is presently in a transition phase due to a change in the principle investigators. The new PI Dr. David Kelvin is continuing some of the previous projects directed toward the molecular and biochemical analysis of chemotactic receptors, while introducing a new area of research on the molecular mechanisms of differential hemopoietic gene regulation. This report covers work completed and/or published by Dr. Kouji Matsushima and colleagues, and introduces newly initiated and studies contained under Dr. Kelvin's direction.

OBJECTIVES

We are pursuing studies of the mechanism underlying the chemotactic response to intercrines by studying the specifications of cytokine receptor interactions, performing biochemical analysis of chemotactic receptors, cDNA cloning of these receptors and signal transduction pathways.

MAJOR FINDINGS

1. Functional, Biochemical and Molecular Properties of MCAF and IL 8

The laboratory has previously purified and molecularly cloned two chemotactic molecules IL 8 and MCAF. The two molecules are members of the Platelet Factor Four super family of cytokine molecules and are each members of different subfamilies distinguished structurally by the C-X-C arrangement in the IL 8 subfamily and the C-C arrangement in the MCAF subfamily. Recent studies have revealed that transcriptional regulation by TNF, IL 1 or PMA of the IL 8 is controlled by cis acting elements located between -94 and -71 of the 5' end of the gene. Multiple factors bind to this region including NF-kB and c/EBP like proteins. Increased levels of IL 8 and MCAF could be induced with PSK, while dexamethasone inhibited the induction of both IL 8 and MCAF gene expression by IL 1 and TNF. Examination of different subsets of lymphocytes for the expression of IL 8 protein or mRNA showed that CD4+ lymphocytes express mRNA

but did not secrete substantial quantities of biologically active IL 8. LGL cells though, can be induced to express IL 8 mRNA and biologically active IL 8 in response to treatment with IL 2 or anti CD16.

Ongoing studies of the three dimensional structure of IL 8 using NMR and X-ray crystallography indicate that regions of the IL 8 molecule that maybe important for receptor binding are located in the amino terminal 9 amino acids and the B-bend located around His-33. The amino terminal structure is extremely flexible and may indicate the presence of a hinge region close by. Chimeric molecules among the IL 8 family are being constructed to investigate the contribution these regions have in receptor binding.

2. Biological and Molecular Characteristics of IL 8 and MCAF Receptors

Peripheral blood mononuclear cells, the monocytic cell line THP-1, and myelocytic cell line cells, U937, were all shown to specifically bind MCAF and express between 1,000 and 15,000 receptors per cell. The nature of receptor turnover is similar to that of IL 8 receptors, in that rapid internalization could be stimulated by MCAF binding. Greater than 80% of the receptors were internalized within 5-10 minutes after MCAF binding. Recycling of receptors was also demonstrated as receptors were re-expressed on the cell surface within 20 minutes of ligand binding. The Kd for binding was approximately 25 nM for monocytes and the THP-1 cell line. Competition studies with various ligands from the MCAF cysteine-cysteine family showed the MIP-1B and I309 were not capable of inhibiting binding, however MIP-1α could inhibit binding by as much as 40% on THP-1 cells and human monocytes. Comparison of the amino acid sequence from these family members indicate that ligand specificity could be dictated by as few as three amino acids.

A soluble MCAF receptor assay has been developed to facilitate the biochemical characterization and purification of the MCAF receptor. Solubilized receptors display similar binding attributes as receptors expressed on intact cells; they can be partially inhibited by MIP-1a but not MIP-1B or I309 and have a binding constant of approximately 30 nM. Preliminary studies indicate the molecular weight of the receptor to be 40k using MCAF crosslinking and SDS-PAGE electrophoresis and 38k as determined by HPLC gel filtration. The receptor ligand complex can bind to ConA indicating the receptor is glycosylated. MCAF ligand affinity columns have been employed to partially purify the soluble MCAF receptor. Current studies are aimed at purifying sufficient quantities of the receptor to determine a partial amino acid sequence.

Scatchard analysis of IL 8 binding to U937 cells which contain up to 20,000 receptors per cell, suggest that these cells contain two distinct IL 8 receptor types. Chemical cross-linking studies using different cell types supports the idea that multiple IL 8 receptor forms exist. Using the chemical cross-linker DFDNB, I125 IL 8 could be specifically cross-linked to receptors (2-7% of total binding could be cross-linked) on the cell surface or on isolated membranes of U937 cells, DMSO induced HL-60 cells, and human neutrophils. Solubilized cells or membranes separated on SDS PAGE gels showed that U937 cells contain two cross-linked species, a very prominent 52 Kd and a

broad 80 Kd species. HL-60 cells induced to differentiate with DMSO displayed an identical pattern, whereas human neutrophils displayed a very different pattern where two distinct bands could be identified at 70 Kd (intense) and 54 kD (weak). Glycosylation studies and cDNA cloning are presently under way to determine if these different IL 8 receptor species are representative of different genes, alternative splicing or post transcriptional modifications. Competition studies with other members in the IL 8 family show that several members can bind to IL 8 receptors on U937 cells. IL 8 and MuMIP-2 compete with equal concentrations, HuMIP-2b is less effective and HuMIP-2a competes 4-6 times less than HuMIP-2b. Exploration of the binding characteristics of IL 8 receptors on human neutrophils are presently under way, and preliminary data indicate that U937 cells can be induced by retinoic acid to express different cross-linked species as well as receptors with different affinities.

These data support the notion that individual IL 8 cytokine family members have unique receptors that preferentially bind a specific ligand with high affinity and other family members with lower affinities.

SIGNIFICANCE

1. Control of the production of IL 8 and MCAF is important for the regulation of the normal inflammatory response. These molecules are under both transcriptional and post-transcriptional control. IL 1, TNF and dexamethasone can control transcription of the IL 8 and MCAF genes, while post-transcriptional control of IL 8 is apparently regulated in a cell type specific manner. Reagents designed to regulate these cytokines may prove effective in treating various inflammatory disorders.

2. The three dimensional structure of IL 8 predicts the amino terminal portion of the molecule may be important for receptor binding. Studies using various ligands in binding experiments show that multiple species of the IL 8 family will bind to the same receptor. Similar findings are true for the MCAF receptors where MIP-1a will also compete for MCAF binding. These results indicate conserved regions in the amino terminal of the IL 8 family or MCAF family are important for ligand binding. Analogues and antogonists can be developed for modulating inflammatory reactions.

3. Multiple biochemical species of IL 8 receptors have been identified. These species correlate with IL 8 receptors with different affinities. The cloning of these receptors will provide definitive proof in identifying the correct ligand of the IL 8 family with the highest affinity for a specific receptor. MCAF receptors also bind other ligands of the same family, indicating receptors binding multiple ligands may be a common feature of the chemotactic receptor family. The soluble receptor assay developed for MCAF will aid in the development of soluble antagonists and agonist for MCAF that may be able to regulate monocyte infiltration of tumors and inflammatory sites.

PROPOSED COURSE

1. Three chemotactic receptors have recently been cloned. The C5a, human FMLP, and rabbit FMLP receptors are all highly conserved and form a subgroup of the rhodopsin super family. They contain seven transmembrane domains and all have a serine theronine rich carboxy terminus. Using the regions of highest nucleotide homology we are presently attempting to clone the IL 8 receptor from a U937 cDNA library and the MCAF receptor from a THP-1 cDNA library using low stringency screening or degenerate oligonucleotide PCR technology. We have been able to isolate several clones with at least one belonging to this receptor family.

2. Construction of hybrid IL 8 and MCAF cytokines using other members of the same family and examining the binding characteristics and biological phenotypes.

3. Biochemical purification of the IL 8 and MCAF receptors.

4. Other members of this receptor family will also be cloned from T-lymphocytes and B-lymphocytes.

5. Identification and sequencing of genomic clones of IL 8 to see if alternative splicing can account for different receptor types.

6. Identification of the signal transduction pathways involved in the chemotactic response.

7. Cloning of genes involved in the tissue specific expression of inflammatory proteins.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09216-11 LMI

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.)*
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	Acting Section Head	IS, LMI, NCI
Others:	G. Cox	Senior Staff Fellow	IS, LMI, NCI
	P. Latham	BTP Fellowship	IS, LMI, NCI
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	D. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*
BCDP, PRI/DynCorp, Frederick, MD (G.L. Gusella, T. Musso, A.D. Brooks); LEI, BRMP, DCT, NCI (R. Wiltrout); Instituto di Microbiologia Medica, Perugia, Italy (E. Blasi); McGill Univ., Montreal, Canada (D. Radzioch).

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 most important and novel aspect that we have analyzed is the response of macrophages and monocytes through the ß-chain of the IL-2 receptor (IL-2$R_ß$) that is constitutively expressed on circulatory cells. In contrast induction of IL-2$R_ß$ by IFN$_γ$ is needed to render murine macrophages susceptible to activation by interleukin 2. Studies on the molecular response elicited by IL-2 in monocytic cells revealed that IL-2 induced augmentation of the IL-2$R_ß$ without affecting IL-2$R_α$ expression. Induction of IL-6 and CSFI receptor was also associated to monocyte activation by IL-2. Synergism between IL-2 and IFNγ or CSFI has been observed in the activation of cytotoxic activity. In contrast, inhibitory effects on the activation by IL-2 are exerted by IL-4 and TGF$_ß$. The interaction between IL-2 and other cytokine is being analyzed at the levels of nuclear transactivating factors. During the course of these studies the modulation by IFN$_γ$ of transactivating factors involved in the expressing of IL-2$R_α$ chain has been established. Moreover, a new DNA binding protein recognizing the HIV-LTR as well as the IL-2 receptor α-chain is being cloned. Studies on the modulation of DNA binding proteins are also in progress to determine the mechanisms by which IFNγ can inhibit macrophage functions (c-fos mRNA expression, induction of IL-6 by IL-1) under certain conditions. Finally we have extensively analyzed the expression and the role of double stranded RNA-dependent enzymes in the activation of murine macrophages. The results are consistent with the hypothesis of a central role for these enzymes in the late phases of macrophage activation, and lead to the discovery of the unique macrophage activating properties of picolinic a metabolite of tryptophan. These results provided the first evidence of a direct role of tryptophan metabolites in macrophage activities.

PROJECT DESCRIPTION

PERSONNEL

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D. L. Longo	Associate Director	OAD, NCI

OBJECTIVES

The objectives of the research have been to study: 1) the cytokine network induced 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. IL 2 Receptor Expression and Effects of IL 2 on Monocytic Cells.

Although convincing information exists that monocytes can respond to IL 2, the nature of the IL 2 receptor on monocytes and the biochemical and molecular basis for this response remain to be elucidated.

We investigated the expression of IL 2R subunits in human monocytes using the TU27 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 but did not block IFNγ-induced cytotoxicity. This data demonstrate that the p75 chain is constitutively expressed on human monocytes and is involved in the activation of monocytes by IL 2. The expression of IL 2Rβ alone is sufficient to mediate the induction of tumoricidal activity by IL 2 alone. We recently determined that murine Mϕ express IL 2R, defined differences between the expression of IL 2R subunits on murine Mϕ versus human monocytes, and established a role for IL 2 as a costimulator of murine Mϕ tumoricidal activity. In order to completely eliminate the possible contributions of even a few contaminating lymphoid cells, we utilized v-raf/v-myc-immortalized murine Mϕ cell lines as a source of homogeneous Mϕ. Binding of radiolabeled IL 2 to the surface of ANA-1 Mϕ followed by chemical crosslinking demonstrated that IL 2Rα and IL 2Rβ are both present on ANA-1 Mϕ constitutively. IFNγ increased the expression of IL 2Rα but did not appear to influence the expression of IL 2Rβ. In contrast to the ability of IL 2 alone to induce the

tumoricidal activity of human monocytes, IL 2 alone (up to 20,000 U/ml) does not induce tumoricidal activity in murine Mϕ cell lines or normal murine peritoneal exudate Mϕ. However, IL 2 (1,000 U/ml) acts synergistically with IFNγ (100 U/ml) to induce Mϕ tumoricidal activity. These results demonstrate the expression of IL 2R subunits on murine Mϕ cell lines and establish the role of IL 2 as a costimulator of Mϕ-mediated killing of tumor cells. That IFNγ plus IL 2 induces tumoricidal activity in C3H/HeJ-derived Mϕ, which are genetically incapable of being activated to tumoricidal activity by IFNγ and/or LPS, indicates that IL 2 circumvents the genetic defect of these Mϕ. This suggests a role for IL 2 as a physiological counterpart of LPS. Different populations of tissue macrophages may respond differently to stimuli of activation. Rat liver macrophages (Kupffer cells) isolated by collagenase liver perfusion and treated in vitro were able, like rat monocytes, to respond to IL 2 alone, but peptone-elicited peritoneal macrophages were not. IL 2 was, in fact, a better stimulus than the combination of IFNγ and LPS. The effect was not related to the presence of natural killer (NK) cells in the liver nonparenchymal cell fractions, since depletion of NK cells by specific antibody and complement did not affect the expression of IL 2-induced cytotoxicity by Kupffer cells. Kupffer cells were found to constitutively express both α and ß chains of the IL 2 receptor, as determined by cross-linking studies, which may explain their rapid and marked response to IL 2.

2. Modulation of Macrophage/Monocyte Gene Expression by IL 2 and IFNγ.

The mechanisms by which IL 2 activated monocytic cells are unknown. Therefore we undertook extensive analysis of the genes whose expression is modulated by IL 2. We investigated the effects of IL 2 and IFNγ on the expression of the proto-oncogene c-fms mRNA, which encodes for the macrophage CSF receptor. Low constitutive expression of c-fms mRNA was detected in fresh human monocytes. Stimulation of monocytes with IL 2 induced a significant increase in c-fms mRNA relative to medium control that was observed as early as 6 hr after IL 2 stimulation. Dose-response experiments showed that 100 U/ml of IL 2 were sufficient to enhance the expression of c-fms mRNA. In contrast IFNγ did not modify the levels of c-fms transcript. Immunoprecipitation experiments demonstrated that IL 2 enhanced c-fms glycoprotein levels. Experiments in which monocytes were activated with IFNγ or IL 2 followed by macrophage CSF-1 and then tested for tumoricidal activity demonstrated that macrophage CSF-1 sustained the cytotoxic activity induced by IL 2 but not by IFNγ. These data demonstrate that IL 2 enhances c-fms mRNA and c-fms glycoprotein expression suggesting that IL 2, by augmenting expression of macrophage CSF-1 receptors, can lead to prolongation of monocyte-mediated tumoricidal activity obtained in the presence of exogenous macrophage CSF-1.

The importance of c-fms activation was evidenced by studies in which monocytic cell lines were transfected with the human c-fms mRNA. Expression vectors containing either normal or oncogenic point-mutated human c-fms genes were transfected into interleukin 3 (IL 3)-dependent 32D cells in order to determine the effects of CSF-1 signaling in this murine clonal myeloid progenitor cell line. CSF-1 was shown to trigger proliferation in association with monocytic differentiation of the 32D-c-fms cells. Monocytic differentiation was reversible upon removal of CSF-1, employing that CSF-1 was

required for maintenance of the monocyte phenotype but not sufficient to induce an irrevocable commitment to differentiation. Human CSF-1 was also shown to be a potent chemoattractant for 32D-c-fms cells, suggesting that CSF-1 may serve to recruit monocytes from the circulation to tissue sites of inflammation or injury. Although c-fms did not release 32D cells from factor dependence, point-mutated c-fms[S301,F969] (Leu-301 → Ser, Tyr-969 → Phe) was able to abrogate their IL 3 requirement and induce tumorigenicity. IL 3-independent 32D-c-fms[S301,F969] cells also displayed a mature monocyte phenotype, implying that differentiation did not interfere with progression of these cells to the malignant state. All of these finding demonstrate that a single growth factor receptor can specifically couple with multiple intracellular signaling pathways and play a critical role in modulating cell proliferation, differentiation, and migration.

Gene expression in IL 2 treated monocytic cells have been compared in murine macrophages and monocytes. IL 2 along induces little or no detectable expression of IL 1ß, IL 6, IL 7, or IFNß mRNA in murine Mϕ. However, IL 2 was a potent inducer of IL 1ß, IL 6 in human monocytes.

3. Regulation of IL 6 Expression in Human Monocytes.

IL 6 is a pleiotropic cytokine that plays an important role in the mediation of the acute phase response, in the regulation of hematopoiesis and immune response. Monocytes are the main source of IL 6, however the effects of some cytokines on IL 6 production are controversial. We investigated the stimulatory and inhibitory signals that control IL 6 production by monocytes. We found that IL 1ß induces IL 6 mRNA expression and bioactivity in elutriated monocytes. TGFß has been shown to inhibit IL 6 production in endothelial cells but also to increase IL 6 production in chondrocytes. Because of this dual role of TGFß on IL 6 production by other cell types we were particularly intrigued by the effect of TGFß on IL 6 production by monocytes. TGFß did not induce IL 6 production in monocytes, moreover TGFß inhibited in a dose dependent manner the induction of IL 6 by IL 1 at the level of mRNA and bioactivity. Human monocytes constitutively express IL 2Rß and the interaction of IL 2 with IL 2Rß seems sufficient to trigger the monocytes activation. Human monocytes respond to IL 2 with peroxide production, microbicidal and tumoricidal activity, induction of IL 1ß and TNF. We were interested to investigate whether IL 2 can effect the expression of IL 6 in monocytes. We found that stimulation of monocytes with IL 2 induced IL 6 mRNA expiration as early as three hrs, in a dose dependent manner. IFNγ, despite its ability to activate monocytes to cytotoxic stage, did not induce IL 6 production leading to the conclusion that IL 6 expression in monocytes is not associated with the cytotoxic phenotype. Since IFNγ did not induce IL 6 in monocytes but can modulate the expression of other genes, we investigated the effects of IFNγ in the induction of IL 6 by IL 1ß or IL 2. We found that IFNγ blocked IL 1ß-induced IL 6 mRNA. No decrease in IL 2-induced IL 6 mRNA was observed in the presence of IFNγ. We showed that TGFß downregulates IL 1ß-induced IL 6 production by monocytes. To address the question whether TGFß, as IFNγ has differentially inhibitory effects, we cultured monocytes in the presence of TGFß plus IL 2. We observed that TGFß significantly decreases IL 6 expression induced by IL 2. In conclusion, TGFß inhibits both IL 1 and IL

2-induced IL 6, on the contrary IFNγ selectively blocks IL 6 induced by IL 1 but not by IL 2. Studies are in progress to understand the mechanisms by which IFNγ is selectively inhibiting IL 6 induction and the differences between the inhibitory effect of TGF and IFNγ.

4. The Suppressive Effects of IL 4 and TGFβ on IL 2 Activity in Monocytic Cells.

Studies were pursued to evaluate the presence of inhibitory signals that could counteract the activating properties of IL 2. We found that IL 4 and TGF_β were capable of inhibiting the activation of cytotoxic macrophages by IL 2. These studies were extended to the analysis of IL 2R mRNA expression in IL 4 and TGF_β treated monocytes. The results demonstrated that the constitutive expression of IL $2R_\beta$ is not affected by either IL 4 or TGF_β. These results suggest that the inhibitory effect of IL 4 and TGF_β do not seem to involve changes in IL 2R expression. In contrast studies on mRNA stability indicate that IL 4 and TGF_β may affect IL 2 receptor gene at a posttranscriptional level. These effects may be associated with the inhibition of IL 2 induced cytotoxicity.

5. DNA Binding Proteins in Human Monocytes.

Human monocytes constitutively express the intermediate affinity p75 IL 2Rβ but not the low affinity p55 IL 2Rα. IFNγ induces IL 2Rα surface expression on monocytes. We investigated the response of the human monocytic cell line U937 to IFNγ at the nuclear level by measuring the changes of IL 2Rα promoter DNA-binding proteins by electrophoretic mobility band shift assays. Nuclear extracts from IFNγ treated, but not medium treated U937 cells, expressed a DNA-binding protein that binds an oligonucleotide comprising the region of IL 2Rα promoter from -270 to -237. This 34bp oligonucleotide contains a kB binding site and a SRE-like binding site. The DNA-protein complex was specific since its formation was inhibited by 100-fold excess of non-labeled 34bp oligomer but not by 100-fold excess of non-labeled SP1 specific oligomer. We also found that nuclear extracts from medium treated U937 cells specifically bind an oligonucleotide comprising the negative regulatory element (NRE) of the IL 2Rα promoter from -406 to -367. Interestingly, nuclear extracts from IFNγ-treated U937 cells displayed a significant decrease in the protein binding to the NRE. These results indicate that modification of binding to the NRE. These results indicate that modification of at least two DNA binding proteins is associated with IL 2Rα expression on U937 cells.

Since the IL 2Rα promoter contains sequences similar to these present in the HIV-LTR, studies were initiated to determine whether monocyte activation could effect the expression of nuclear proteins recognizing HIV-LTR. We studied the macrophage DNA-binding proteins using, in band shift assays, the 219 bp probe (-339 to-120) comprising the Negative Regulatory Element (NRE) of HIV-LTR. We demonstrated that in the macrophage cell line GG2EE nuclear proteins constitutively expressed binding this region of the HIV-LTR and LPS induced the expression of an LPS-responsive factor in a protein synthesis-dependent process. The binding of the LPS-responsive factor to the NRE was specific and evident after six hrs of stimulation of the macrophages with LPS. Detailed analysis of fragments of the 219 bp probe that the LPS-responsive factor

recognizes a region between -216 to -158 of the HIV-1 LTR which interestingly shares homology with a region of the NRE of IL 2Rα promoter. Nuclear extracts from elutriated human monocytes showed a binding similar to the one obtained with GG2EE cells. The LPS-inducible HIV-LTR binding protein can be part of the control of HIV virus expression during monocytes activation and can be important in the regulation of LPS-inducible genes in monocytes. Characterization of functional properties and molecular cloning of the protein by screening an expression library from LPS activated monocytes are in progress.

6. Expression of Early Response Genes (c-fos and c-fm) During Cell Activation.

It is well established that c-gm and c-fos can control gene expression through the formation of the nuclear factor AP-1. We have previously observed that the mechanisms regulating c-fos expression in macrophages differs from those reported for fibroblasts. Therefore it was important to determine how macrophage activation can modulate c-fos and/or c-fm expression.

Studies on fibroblasts have shown that the members of the ras oncogene family can modulate c-fos expression. We analyzed the induction of c-fos mRNA and protein by the protein kinase C (PKC) activator 12-0-tetradecanoyl-phorbol-13-acetate (TPA) in exponentially growing NIH3T3 fibroblasts transformed by transfection with ras oncogenes. We found that H-ras has the unique ability to inhibit c-fos induction by TPA. In contrast, normal c-fos expression was induced by TPA in fibroblasts transformed by N- or K-ras or by the ras-unrelated oncogenes dbl and trk. The inhibition of c-fos induction by H-ras was not due to alteration in the binding of TPA to the transformed cells or to the selection of idiosyncratic clones. These results provide clear evidence that H-ras is functionally different from K- or N-ras and that different cell types in which ras genes may be differentially express may express variable levels of c-fos.

To establish the mechanisms controlling c-fos expression in murine macrophage experiments were performed in which the effects of interferon γ (IFNγ) on c-fos mRNA expression in fresh peritoneal macrophages and in the immortalized macrophage cell line ANA-1 were investigated. Treatment of macrophages with IFNγ strongly decreased the induction of c-fos mRNA by 12-0-tetradecanoylphorbol 12-acetate (TPA), lipopolysaccharide (LPS) or calcium ionophore A23187 (CaI) in macrophages. Under the same experimental conditions, IFNγ induced alogia synthetase mRNA and did not affect the constitutive expression of TGFß mRNA indicating that IFNγ did not induce general degradation of mRNAs. Run off experiments with the ANA-1 macrophages indicated that c-fos was constitutively transcribed at low levels and that TPA augmented c-fos transcription. However, IFNγ did not inhibit constitutive or TPA-induced c-fos transcription. These results indicated also that IFNγ treated macrophages are fully responsive to TPA. The stability of c-fos mRNA evaluated in IFNγ treated macrophages following the block of RNA synthesis by DAct was decreased in comparison to the stability of c-fos mRNA in untreated macrophages with IFNγ macrophages. These results indicated that the IFNγ affected c-fos mRNA augmentation by TPA on the posttranscriptional level.

IFNγ effect may represent very important physiological control mechanism of c-fos proto- oncogene expression in macrophages.

The analysis of the effects of IFNγ on c-fos mRNA expression was extended to human monocytes. We have not demonstrated that IFNγ is able to inhibit the expression of c-fos and c-jun in human monocytes within 30 min when fos mRNA was induced by serum, LPS, or CSF-1. The inhibitory effect of IFNγ on c-fos appeared to be stimulus-specific, since IFNγ could not inhibit fos mRNA induced by phorbol ester. The inhibitory effect of IFNγ to LPS-induced c-fos was not generalized to other LPS-induced gene expression, since no inhibitory effect was seen on the expression of LPS-induced IL 1β. The inhibitory effect of IFNγ on c-fos was not related to its ability to stimulate cytotoxicity in monocytes, since IL 2, which can also induce cytotoxicity in monocytes, had no effect on expression of c-fos. The inhibitory effect of IFNγ was found to be at the transcriptional level of gene expression by nuclear run-on assay. Modulations in c-fos and c-jun can affect the formation of the AP-1 DNA binding protein and so influence the expression of genes responding to AP-2. Studies are not in progress to determine if IFNγ can modulate the formation and transcription of AP-1 enhancer elements. Further studies will also explore the mechanism of the inhibitory effect of IFNγ on c-fos.

7. Double-Stranded RNA-Dependent Enzymes in Macrophage Activation: The Tryptophain Connection.

Previous work in the Immunobiology Section has indicated that the double-stranded-RNA-dependent protein kinase (dsRNApK) and/or the 2′-5′-oligoA synthetase (20ASE) can be involved in the process of macrophage activation to a cytotoxic stage. Therefore, it was important to examine the expression of these enzymes following exposure of macrophages to activating signals.

The expression of dsRNApk has been analyzed in murine macrophages following activation with LPS and/or IFNγ. The dsRNApk is an IFN-inducible enzyme whose activity has been involved in the antiviral response elicited by IFNs. Upon activation in the presence of deRNA the deRNApk phosphorylates the eIF1 a subunit preventing the recycling of this factor in the complex initiating protein synthesis and consequently leading to the general inhibition of translation. We showed that macrophages express a functional dsRNApk. In murine macrophages both LPS and IFN induced the expression of the deRNApk measured as the ability of this enzyme to phosphorylate exogenous histones in the presence of synthetic dsRNA, poly[I:C].

LPS increased the expression of dsRNApk at levels comparable to those elicited by IFNγ. Both LPS and IFNγ trigger macrophage tumoricidal activity. The mechanism and the pathways leading to macrophage activation are not fully understood. In order to determine a possible role of the dsRNApk in the expression of the tumoricidal activity of macrophages we used the protein kinase inhibitor, 2-aminopurine (2AP), which presents a relative specificity for the dsRNApk. Addition of 2AP (2mM) selectively blocked macrophage cytotoxic response induced by LPS whereas the activation elicited by LPS plus IFNγ was not affected. The lower sensitivity of macrophages cytotoxicity, induced by LPS plus IFNγ, to 2 AP is not due to a higher expression of the

deRNApk. In fact, the costimulation of macrophages with LPS plus IFNγ did not result neither in the synergistic nor additive induction of dsRNApk. At the concentration used to inhibit macrophage cytotoxicity, 2AP does not affect other serinethreonine kinases involved in signal transduction such as PKC and PKA, as assessed by the ability of PMA and forskolin to enhance c-fos mRNA expression in the presence of 2AP. This evidence indicates that, under our conditions, 2AP does not act as a general kinase inhibitor and strengthens the possibility for involvement of the dsRNApk in the control of specific cellular mechanisms. Indeed, data have been reported showing the role of dsRNApk in the translatability of exogenous genes. To investigate this possibility we analyzed the expression of different genes which are involved in the inflammatory response and are essential part of the cytokine network. Gene expression was affected both negatively and positively by 2AP although the negative effects were much more dramatic. In response to stimulation with LPS or LPS/IFNγ, expression of TNFα and CSF-1 mRNAs was completely downregulated by 2AP (indicating that TNFα is not required for cytocidal activity) whereas the induction of IL 1 and IL 6 mRNAs was slightly enhanced.

Our data also indicate that also the mechanisms of induction of different cytokines in macrophages may be distinguished by susceptibility to 2AP and suggest a possible role of the dsRNApk in the control of gene expression and cellular functions. dsRNApk role on translation could be a mean to control the translation of proteins required for the expression or regulation of specific genes.

It was previously suggested that ribosomal RNA precursors, that accumulated in cytotoxic macrophages, may fold into secondary structures containing stable double stranded regions. It was further suggested that these double stranded regions can activate double stranded RNA dependent enzymes and lead to cytotoxicity. These hypothesis were supported by studies on the effect of picolinic acid macrophage activation. We have studied the effects of picolinic acid, a product of tryptophan degradation, on the activation of mouse peritoneal macrophages (Mϕ). Picolinic acid acts synergistically with IFNγ in activating Mϕ from C57BL/6 mice. Moreover, Mϕ from C3H/HeJ mice and C3H/HeN that do not become cytotoxic in response to IFNγ alone could be fully activated by exposure to picolinate plus IFNγ. These results indicate that picolinic acid is a potent costimulator of Mϕ activation that functions as a second signal. Inasmuch as we have previously demonstrated that the activation of cytotoxic Mϕ correlates with specific changes in ribosomal RNA (rRNA), we investigated whether picolinic acid could modify Mϕ RNA metabolism. Picolinic acid inhibited the synthesis of total Mϕ RNA, the accumulation of newly synthesized 28S rRNA, and augmented the steady state levels of rRNA precursors (pre-rRNA). These changes in RNA metabolism were similar to those previously described in murine Mϕ activated in vitro or in vivo to express tumoricidal activity. These results demonstrate that picolinic acid is a potent biologic Mϕ second signal, suggest that the changes in rRNA are causally connected with the expression of tumoricidal activity, and suggest that existence of an autocrine effect mediated by picolinic acid.

FUTURE COURSE

The future courses of the various projects are listed at the end of the project description for simplicity and clarity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09359-01 LMI

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.)*: Modulation of Macrophage Gene Expression and Functions

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

PI:	G. W. Cox	Senior Staff Fellow	IS, LMI, NCI
Others:	L. Varesio	Visiting Scientist	IS, LMI, NCI
	J. J. Oppenheim	Chief	IS, LMI, NCI
	J-M. Wang	Special Volunteer	IS, LMI, NCI

COOPERATING UNITS *(if any)*: Laboratory of Experimental Immunology, Frederick, MD (B. Mathieson); Program Resources, Inc., Frederick, MD (S. Giardina); Chittaranjan National Cancer Institute, Calcutta, India (U. Chattopadhyay)

LAB/BRANCH: Laboratory of Molecular Immunoregulation

SECTION: Immunobiology Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

The role of IFNγ plus IL 2 in the activation of murine macrophages (Mϕ) was investigated. The Mϕ cell line ANA-1 does not express detectable levels of mRNA for the p55 subunit of the IL 2 receptor (IL 2Rα) constitutively. However, ANA-1 Mϕ express low levels of the p70-75 subunit of the IL 2 receptor (IL 2Rß) on their cell surface, and treatment of these Mϕ with IFNγ induces the expression of IL 2Rα mRNA and protein. Mϕ cell lines and normal murine peritoneal Mϕ do not express constitutive cytotoxic activity against p815 mastocytoma cells in vitro. Moreover, these Mϕ, in contrast to normal human monocytes and rat Kupffer cells, do not become tumoricidal after treatment with IFNγ alone or IL 2 alone. However, Mϕ become activated after coincubation with IFNγ plus IL 2. Studies designed to identify inhibitors of Mϕ tumoricidal activity revealed that IL 4 inhibits IFNγ plus IL 2-induced but not IFNγ plus LPS-induced cytotoxicity. At least one mechanism of IL 4 suppressive activity appears to be its ability to reduce the IFNγ-induced expression of IL 2Rα mRNA in Mϕ. Additional studies indicated that IFNγ plus IL 2-treated Mϕ are induced to express several genes that encode chemotactic cytokines (e.g. JE, KC, and CSF-1).

Surprisingly, murine Mϕ cell lines activated by IFNγ express several genes that are homologous to murine IL 2. A molecular cloning approach was utilized to explore the expression of novel IFNγ-inducible genes in murine Mϕ. A cDNA library was prepared using mRNA isolated from an IFNγ-treated Mϕ cell line. Several cDNA clones were isolated from the library using a fragment of the murine IL 2 cDNA as a probe. Sequence analysis indicated that two of these clones represent novel genes.

PROJECT DESCRIPTION

PERSONNEL

George W. Cox	Senior Staff Fellow	IS, LMI, NCI
Luigi Varesio	Visiting Scientist	IS, LMI, NCI
Joost J. Oppenheim	Chief	IS, LMI, NCI
Ji-Ming Wang	Special Volunteer	IS, LMI, NCI

OBJECTIVES

Overall, these studies are designed to define how cytokines regulate macrophage (Mϕ) tumoricidal activation. One goal of these studies is to characterize the biologic role of IL 2 in the activation of Mϕ. To achieve this, we have examined the ability of IL 2 to modulate cytokine gene expression in Mϕ, and have evaluated the influence of IL 2 on the tumoricidal capacity of Mϕ. Another goal is to identify and clone novel genes that are expressed in cytotoxic Mϕ activated by IFNγ and/or IL 2. We have pursued this by using Northern blot, PCR, cDNA library construction and screening, and DNA sequencing techniques.

MAJOR FINDINGS

1. Expression and Function of IL 2 Receptors in Murine Mϕ

We and others have previously established that normal human monocytes are activated for tumor cell lysis after exposure to IL 2 alone. It has been demonstrated that human monocytes express constitutive levels of the p70-75 subunit of the IL 2 receptor (IL 2Rß) but not of the p55 subunit of the IL 2 receptor (IL 2Rα). However, treatment of human monocytes with IFNγ results in their expression of IL 2Rα mRNA and surface protein. The expression of IL 2Rß alone is sufficient to mediate the induction of tumoricidal activity by IL 2 alone. Whether human or murine Mϕ or murine monocytes also express functional IL 2R subunits was unknown for some time. We recently determined that murine Mϕ express IL 2R, defined differences between the expression of IL 2R subunits on murine Mϕ versus human monocytes, and established a role for IL 2 as a costimulator of murine Mϕ tumoricidal activity.

In order to completely eliminate the possible contributions of even a few contaminating lymphoid cells, we utilized v-raf/v-myc-immortalized murine Mϕ cell lines as a source of homogeneous Mϕ. We previously reported the establishment and characterization of the murine Mϕ cell lines GG2EE and ANA-1. These Mϕ cell lines are particularly useful for these studies because they are cloned populations of Mϕ that do not require exogenous growth factors for their long-term survival and proliferation, and they are derived from strains of mouse that are either hyporesponsive (GG2EE) or responsive (ANA-1) to LPS. Therefore, the effects of IL 2 can be examined without regard for the potential existence of extraneous endotoxin.

In collaboration with Drs. B. Mathieson (LEI, NCI) and S. Giardina (BCDP, PRI), we initially observed that ANA-1 Mϕ do not constitutively express detectable levels of IL 2Rα mRNA. However, a brief (5 hr) exposure to 100 U/ml of IFNγ is sufficient to induce IL 2Rα mRNA in ANA-1 Mϕ. Flow cytometric analysis of anti-IL 2Rα monoclonal antibody binding studies revealed that ANA-1 Mϕ express low constitutive levels of IL 2Rα on their cell surface that are augmented by an 18 hr treatment of these Mϕ with 100 U/ml of IFNγ. Binding of radiolabeled IL 2 to the surface of ANA-1 Mϕ followed by chemical crosslinking demonstrated that IL 2Rα and IL 2Rß are both present on ANA-1 Mϕ constitutively. IFNγ increases the expression of IL 2Rα but does not appear to influence the expression of IL 2Rß. In contrast to the ability of IL 2 alone to induce the tumoricidal activity of human monocytes. IL 2 alone (up to 20,000 U/ml) does not induce tumoricidal activity in murine Mϕ cell lines or normal murine peritoneal exudate Mϕ. However, IL 2 (1,000 U/ml) acts synergistically with IFNγ (100 U/ml) to induce Mϕ tumoricidal activity. Our results demonstrate the expression of IL 2R subunits on murine Mϕ cell lines and establish the role of IL 2 as a costimulator of Mϕ-mediated killing of tumor cells. Moreover, our findings indicate that murine Mϕ differ from human monocytes in that they express both subunits of the IL 2R constitutively and require an additional stimulus along with IL 2 for the induction of cytotoxicity. This suggests the hypothesis that the differentiation of monocytes to Mϕ is associated with a reduced responsiveness to IL 2 or the requirement for additional intracellular signals or pathways that lead to complete activation.

That IFNγ plus IL 2 induces tumoricidal activity in C3H/HeJ-derived Mϕ, which are genetically incapable of being activated to tumoricidal activity by IFNγ and/or LPS, indicates that IL 2 circumvents the genetic defect of these Mϕ. This suggests a role for IL 2 as a physiologic counterpart of LPS. Therefore, existing physiologic mechanisms that lead to the production of IL 2 may serve as additional pathways for Mϕ activation in vivo, and may explain the reason why the genetically compromised C3H/HeJ mouse is apparently no more susceptible to neoplastic disease than the normal C3H/HeN mouse.

2. The Suppressive Effects of IL 4 on IL 2 Activity in Mϕ

In order to be able to pursue an investigation into the mechanism(s) of action of IL 2 in the generation of cytotoxic Mϕ, we decided to identify an inhibitor of IL 2-dependent tumoricidal activity. In collaboration with Drs. J. Oppenheim (LMI, NCI) and U. Chattopadhyay (Guest Researcher for 3 months from Chittaranjan NCI of India), we established that IL 4 does not induce tumoricidal activity in several oncogene-immortalized murine Mϕ cell lines either by itself or in combination with IL 2, LPS or IFNγ. Moreover, IL 4 inhibits the IFNγ plus IL 2- but not IFNγ plus LPS-induced tumoricidal activity of these Mϕ. The effect of IL 4 is not restricted to v-raf/v-myc-immortalized Mϕ cell lines because similar effects are observed using a Mϕ cell line that was established from a spontaneous histicytic sarcoma. The suppressive activity of IL 4 on the ANA-1 Mϕ cell line is both dose- and time-dependent. IL 4 concentrations of 12 to 200 U/ml are sufficient to reduce or completely inhibit IFNγ plus IL 2-induced cytotoxicity, and IL 4 must be present throughout the entire period of time that the Mϕ are incubated with

IFNγ plus IL 2. The effect of IL 4 is specifically mediated by IL 4 rather than a potentially suppressive contaminant of the recombinant preparation because its effects are completely neutralized by the addition of anti-IL 4 monoclonal antibody. IL 4 decreases the ability of IFNγ to induce the expression of IL 2Rα mRNA. Therefore, at least one mechanism by which IL 4 may inhibit IFNγ plus IL 2-induced tumoricidal activity is by reducing Mϕ IL 2Rα mRNA expression. We previously reported that picolinic acid, a tryptophan metabolite, is a costimulator of Mϕ tumoricidal activity. We now find that IL 4 also inhibits IFNγ plus picolinic acid-induced cytotoxicity in ANA-1 Mϕ. We hypothesize that IL 2 and picolinic acid may have a common mechanism of action that is susceptible to IL 4 suppression, and are pursuing this possibility experimentally.

Our results suggest that T_H1 cells can promote the activation of cytotoxic Mϕ in vivo by providing IFNγ and IL 2, whereas T_H2 cells can abrogate Mϕ tumoricidal activity by secreting IL 4. It is clear that the activation of cytotoxic Mϕ in vitro may require multiple agents such as IFNγ plus LPS. We have demonstrated that in addition to LPS, IL 2 and picolinic acid can also serve as costimuli with IFNγ for the activation of cytotoxic Mϕ. That IL 4 inhibits both IL 2- and picolinic acid- but not LPS-dependent cytotoxicity provides an indication that distinct mechanisms may exist by which these agents can act as costimuli with IFNγ for the induction of Mϕ tumoricidal activity. These mechanisms may be distinguished on the basis of their sensitivity to the inhibitory effects of IL 4.

3. Inhibition of Immortalized Murine Mϕ Cell Line Proliferation by IL 2

Because IL 2 induces lymphocyte proliferation, it was of interest to determine whether IL 2 influences the proliferative capacity of Mϕ cell lines. Inasmuch as IFNγ induces the expression of IL 2R on Mϕ cell lines, initial experiments examined the effect of IFNγ on the proliferation of these cells. IFNγ has been shown to suppress the proliferation of several types of cells and established cell lines. Similarly, we have found that IFNγ reduces the proliferation of ANA-1 Mϕ in vitro. IL 2 alone does not influence ANA-1 Mϕ proliferation. Interestingly, however, the addition of IL 2, at concentrations that are sufficient to act as costimuli with IFNγ for the induction of tumoricidal activity, synergize with IFNγ for the inhibition of ANA-1 Mϕ proliferation. IL 4 reverses the suppression of Mϕ cell line proliferation triggered by IFNγ plus IL 2. These results suggest that IL 2 may be beneficial for suppressing the growth of Mϕ tumors in vivo. In addition, IL 2 and IL 4 may be involved in the generation or expansion of Mϕ precursors from the bone marrow. We are pursuing our observations and their implications.

4. IL 2 Induction of Gene Expression in Mϕ

In addition to its ability to induce tumoricidal activity in Mϕ, IL 2 may regulate other functions of Mϕ, and the production and/or secretion of Mϕ-derived cytokines. Studies have been performed to determine the effect of IL 2 on gene expression in Mϕ. RNA was isolated from murine Mϕ that were incubated with medium alone, IFNγ alone, IL 2 alone or a combination of IFNγ

plus IL 2. Northern blot analysis was performed using cDNA probes specific for the gene of interest. For increased sensitivity in detecting certain genes, RNA was reverse transcribed to cDNA, and specific cDNA was amplified by the PCR using gene-specific synthetic oligonucleotide primers. IL 2 alone induces little or no detectable expression of IL 1ß, IL 6, IL 7, or IFNß mRNA in murine Mϕ. However, the addition of IL 2 to IFNγ-treated Mϕ causes an augmentation in the expression of IL 1ß and IL 6 mRNA. In addition, IL 2 alone or in combination with IFNγ induces or augments the expression of genes that encode chemotactic proteins (e.g. KC, JE and CSF-1). In collaboration with Dr. J-M. Wang (LMI, NCI) we have determined that culture supernatants from IL 1- and/or IFNγ plus IL 2-treated Mϕ exhibit chemotactic activity for murine Mϕ cell lines and normal murine peritoneal Mϕ. The levels of chemotactic activity are higher than those found in culture supernatants from medium- or IFNγ-treated Mϕ. Identification and further characterization of the chemotactic activity in IL 2-treated Mϕ culture supernatants is in progress. The IL 2-mediated expression of genes encoding chemotactic proteins may be an important event during the generation of IL 2-dependent Mϕ tumoricidal activity.

5. Cloning of Novel IFNγ- and IL 2-Inducible Genes in Mϕ

The identification of genes expressed during the manifestation of IFNχ- and/or IL 2-dependent tumoricidal activity is of biologic and therapeutic interest because they may encode important intercellular or intracellular proteins (e.g. enzymes or cytokines) relevant in Mϕ-mediated tumor cell killing. During the course of cytokine gene expression studies, Northern blot analyses revealed that the full-length murine IL 2 cDNA hybridizes to several mRNA transcripts that are expressed constitutively in the murine Mϕ cell lines ANA-1 and GG2EE. Because normal Mϕ as well as these Mϕ express a gene(s) that is at least partially homologous to murine IL 2. ANA-1 or GG2EE Mϕ that are incubated with 100 U/ml of IFNγ for 18 hr express augmented levels of at least one of the mRNA species. This mRNA species is also expressed in thioglycollate-elicited mouse peritoneal Mϕ but not in T cell lines (CTLL-2 or EL-4) or fibroblasts (3T3). Northern blots probed with restriction enzyme-digested fragments of the murine IL 2 cDNA or PCR-generated probes reveal that the mRNA transcripts detected in the Mϕ cell lines are homologous to sequences contained within exon I of the murine IL 2 cDNA. We prepared a cDNA library from IFNγ-stimulated GG2EE Mϕ and screened the library using a PCR-generated probe that is identical to base pairs 108-236 of the murine IL 2 cDNA. To date, three cDNA clones have been isolated, sequenced, and appear to represent novel genes on the basis of computer database searches. At least one cDNA contains a relatively unique repeated nucleuc acid sequence that is found in murine IL 2 and several other functional unrelated eukaryotic genes. The genes containing the tandem-repeat sequence (CAG) encode important secreted or cell-associated proteins (e.g. IL 2, glucocorticoid and androgen receptor, and potential transcription factors or homeobox proteins). The expression of these genes and their encoded proteins will be pursued and their role in Mϕ tumoricidal activity will be explored. RNA has also been isolated from Mϕ treated with IL 2 and will be used to generate a cDNA library. This library will be screened for the purpose of identifying and cloning novel genes in IL 2-activated cytotoxic Mϕ.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09251-09 LMI

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.)*

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	LS, LMI, NCI
Others:	N. C. Lohrey	Microbiologist	LS, LMI, NCI
	S. Nishimura	Guest Researcher	LS, LMI, NCI
	F. Tao	Visiting Fellow	LS, LMI, NCI

COOPERATING UNITS *(if any)*

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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.)*

To better understand the effects of host cells 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, and restricted viral expression for both HTLV-I and HIV-I. In HTLV-I infected B cells from acute T cell leukemia (ATL) patients, very few 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 poorly expressed. Also, the macrophage, a reservoir of HIV-I infection in AIDS patients, was shown to be able to restrict HIV-I expression. THP-1, a macrophage cell line, was permissive for HIV-I expression. However, these infected cells become naturally non-productive for HIV 4-6 weeks after infection. Two classes of restricted HIV-I expression were seen: 1) low-level which can be regulated by factors in the nuclei of infected cells and 2) no expression which may be due to methylation of the LTR. Both viruses make proteins which act in trans to positively regulate viral transcription. In these latently infected cultures, transactivation of viral transcription is inhibited. This indicates that these host 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

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MAJOR FINDINGS

1. 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. After 10-17 days, all 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); or 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 surveillance 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.

2. 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.

3. 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. Furthermore, the addition of antibodies specific for either NF-kB or c-rel protein (members of the family of transcription factors) to binding complex show that only the NF-kB antisera inhibited gel shift mobility suggesting that the inhibition blocked NF-kB binding and not c-rel binding. 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.

4. 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 γ-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.

5. 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.

6. Interaction of Human Cytomegalovirus (HCMV) with HTLV and HIV-infected Lymphocytes

Amongst the other human viruses also known 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.

7. Baicalin, a Flavonoid Compound, is a Potent Inhibitor of Both HIV and HTLV Expression

Baicalin, 7-D-glucuronic acid-5,6-di hydroxyflavone, is a traditional Chinese medicinal herb. We have found that it produces a concentration dependent inhibition of HIV-1 infectivity as measured in a syncytium assay using CEM cells, an HIV core protein p24 antigen capture Elisa from infected fresh peripheral blood lymphocytes. Baicalin was very potent in inhibiting the

enzymatic activity of purified HIV-1 reverse transcriptase in an in vitro assay. At the concentrations where optimal inhibition of viral replication was seen, neither cytotoxic nor cytostatic effects on the cells were observed. In chronically infected HTLV-I cells Baicalin inhibited greater than 80% HTLV-I p19 released from the cells as well as inhibiting the enzymatic activity of HTLV-I reverse transcriptase.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09264-09 LMI

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.)*
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:	C. M. Dubois	Visiting Fellow	LMI, NCI
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COOPERATING UNITS *(If any)* LMI, BRMP, DCT, NCI (J. Oppenheim); OAD, BRMP, DCT, NCI (D. Longo); LEI, BRMP, DCT, NCI (J. Ortaldo, R. Wiltrout); BCDP, PRI/Dyncorp, Frederick, MD (L. Falk, M. Birchenall-Roberts, K. Hestdal, J. Rossio, J. Keller, J. Kasper, K. Sill, A. Ochoa); Oncogen, Inc. (T. Purchio); Celltrix, Inc. (L. Ellingsworth).

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	.75	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.)*

In studying the humoral regulation of lymphohematopoietic cell growth and differentiation as well as mature cell function, we have found that transforming growth factor ß has potent bifunctional effects. TGF ß1 and TGF ß2 are equipotent selective inhibitors of hematopoiesis that halt the growth of early human and murine progenitors, but not more differentiated progenitors. Using partially purified murine hematopoietic stem cells in single cell assays with several cytokines, it was shown that TGF ß acted directly on the cells to block growth. In contrast, addition of TGF ß to GM-CSF stimulated bone marrow cells greatly augmented growth, leading predominantly to an increase in granulocytes, and was also shown to have a direct effect in single cell assays. Both in vivo and in vitro experiments with TGF ß show that the hematopoietic stem cells are reversibly prevented from entering the cell cycle. These growth modulatory effects function at, at least, two levels: 1) trans-down modulation of the cell surface receptors for positive regulatory signals and/or 2) interfering with post-receptor signalling of these molecules by decreasing cytokine mediated phosphorylation or by inhibiting specific gene expression. Leukemic cell lines could be either sensitive or insensitive to TGF ß mediated growth inhibition. Growth of neoplastic B lymphocytes can occur by escaping from a TGF ß mediated autocrine inhibitory loop. Activation signals (e.g. phorbol esters) can inhibit tumor cell growth by stimulation of active TGF ß production and induction of cell surface expression of functional TGF ß receptors. In studying effector cell function by CD3+ and CD3- lymphocytes, it was found that cell mediated cytotoxicity was much more sensitive to TGF ß mediated inhibition than cell growth and that expression of the IL 2 receptor p55 chain was inhibited by TGF ß while expression of p75 IL 2 receptor chain was not.

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PROJECT DESCRIPTION

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MAJOR FINDINGS

1. Direct Inhibitory or Stimulatory Effect of TGF ß on Primitive Hematopoietic Cell Growth

We have previously shown that TGF ß selectively inhibits both human and murine hematopoietic progenitor cell growth. In all these assays, we have observed that TGF ß1, TGF ß2 and TGF ß3 are potent inhibitors of hematopoiesis. Regardless of the cytokine(s) used to induce multipotent colonies such as HPP-CFU and CFU-GEMM, colony formation was inhibited by TGF ß whereas formation of CFU-M and CFU-G colonies was not inhibited. As a result of the pleiotropic nature of TGF ß, it is possible that these effects were indirect. To study this, highly purified populations of murine hematopoietic progenitor cells were obtained by separating lineage negative (Lin-) Thy-1+ cells using monoclonal antibodies and fluorescent activated cell sorting. The purified Lin-Thy-1+ cells were plated as single cells in Terasaki plates with IL 3 with and without synergistic cytokines. In these cultures, 1 out of 2.3 cells proliferated under the best conditions. Upon addition of TGF ß, 1 out of 18 cells proliferated showing that 80% of the cells were inhibited and proving a direct effect of TGF ß. Since TGF ß and GM-CSF synergize in stimulating granulopoiesis, single cell experiments were performed using this combination. GM-CSF and TGF ß increases single cell growth of Lin- Thyl- cells but not Lin-Thyl+ cells suggesting a direct effect of TGFß on a novel progenitor.

In addition, 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 cell enabling them to respond to a combination of CSF-1 and IL 3, probably by stimulating an increase in specific receptors. The expansion of these stem cells with HPP-CFU potential is also inhibited by TGF ß. However, the addition of a 25-fold excess of all three positive signals overcomes the inhibitory action of TGF ß. Thus, the activation state of any cell is a composite of the amount of positive and negative signals received.

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

The mechanisms by which TGF ß acts as a potent inhibitor of the growth and functions of lymphoid and hematopoietic 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 ß could be mediated by inhibition of growth factor receptors. TGF ß 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 ß 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 ß 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 ß is a potent inhibitor of the stimulatory effect of many hematopoietic growth factors, in addition to IL 1, we examined the effect of TGF ß on cell surface receptor expression for CSF. TGF ß 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 ß. The TGF ß 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 ß induced reduction in CSF receptor numbers preceded TGF ß's growth inhibitory action. In addition, the ED50 (1 to 10 pM) for TGF ß's CSF receptor modulatory and antiproliferative effect was similar. The effect of TGF ß 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 ß treatment. These data suggest that TGF ß down-regulates the growth of hematopoietic progenitor cells by reducing the expression of growth factor receptors.

The mechanism by which TGF ß blocks the expression of cytokine receptors is not clear. One possibility is that TGF ß can block the transcription of mRNA for these receptors. Using CD3- LGL, we have found that TGF ß can block the transcriptional activation of the p55 chain of the IL 2 receptor but not the p75 chain of the IL 2 receptor.

3. Stimulation of Granulopoiesis by TGF ß: Identification of a Novel Hematopoietic Progenitor Cell

TGF ß1 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 ß 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 ß1 alone exerts no growth stimulatory or inhibitory effect. Morphological examination of colonies demonstrated an increase in granulocyte colonies. In suspension culture, TGF ß1 and GM-CSF stimulated 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 demonstrated a 2- to 5-fold increase in the frequency of progenitor cells that responded to GM-CSF plus TGF ß1 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 ß1 while either factor alone had no effect. TGF ß upregulated the number of GM-CSF receptors on bone marrow cells cultured in GM-CSF. Thus, TGF ß1 can act as a bifunctional mediator of hematopoietic cell growth, and TGF ß1 and GM-CSF act together to stimulate granulopoiesis as measured by large granulocyte colony formation, tentatively designated burst forming unit granulocyte.

Preliminary experiments have shown that the sequential injection of TGF ß1 followed by injection of GM-CSF 40 hrs later (when there is a maximum upregulation of GM-CSF receptors on bone marrow progenitor cells) results in acceleration of myelopoiesis.

4. TGF ß1 Receptor Modulation During Hematopoietic Cell Growth and Differentiation

TGF ß1 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 ß1 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 ß1 receptors during hematopoiesis showed that freshly isolated, granulocyte-depleted, murine and human bone marrow cells exhibited little or no specific TGF ß1 receptor expression. However, culturing of human bone marrow progenitors in GM-CSF or IL 3 for 7 days resulted in the development of mature granulocytes and monocytes which expressed predominantly the 65 kDa TGF ß1 receptor. Examination of the development and modulation of TGF ß1 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 ß1 receptor species was increased on murine CSF-1-derived macrophages but not on GM-CSF-derived macrophages. In addition, IFN γ treatment resulted in a dose and time-dependent down-modulation of TGF ß receptor expression on peritoneal exudate macrophages. The results indicate that freshly isolated hematopoietic cells exhibit differential TGF ß1 receptor expression. Furthermore, as bone marrow mononuclear cells mature in vitro, they acquire TGF ß receptor expression which can be subsequently down-modulated by activation cytokines. These data suggest that receptor modulation by growth factors or activation/differentiation cytokines results in a potential feedback loop by which cellular response to TGF ß1 are controlled.

5. Growth 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 TGF β significantly inhibited proliferation and induced monocytic differentiation of a small percentage of U937 cells. Combinations of TGF β and tumor necrosis factor a (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), and interferon-γ (IFN γ). Examination of TNF a receptor expression on HL60 and U937 cells showed that these cell lines expressed comparable levels of high-affinity TNF binding sites. Treatment of HL60 and U937 cells with TGF β1 did not induce significant changes in TNF a 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 HL60 was studied. Treatment of HL60 wth RA for 7 days has previously been shown to inhibit proliferation and stimulate granulocytic differentiation. Untreated HL60 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 treatment for seven days with suboptimal concentrations of HL60 (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 HL60 differentiation.

6. 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 and 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-13-acetate (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 ß, however, PMA induced increased expression of TGF ß mRNA and, more importantly, release of active TGF ß. A neutralizing antibody to TGF ß was able to reverse the PMA-induced growth inhibition and addition of exogenous TGF ß reversed the effects of the neutralizing antibody. Thus, TGF ß can inhibit human lymphoma cell growth in vitro through an autocrine mechanism. Some lymphoma cells appear to have escaped from TGF ß negative regulation by failing to express functional TGF ß receptors and/or failing to secrete active TGF ß. One mechanism by which PMA acts to inhibit lymphoma cell growth is by inducing the expression of TGF ß receptors and the secretion of active TGF ß thereby reestablishing an autocrine growth inhibitory loop.

On the other hand, we have found that TGF ß inhibited the PMA induced malignant transformation of mouse JB6 epithelial cells. In this system, PMA markedly decreased the expression of TGF ß receptors. As in HL60, addition of retinoic acid stimulated the expression of TGF ß receptors (see J. J. Oppenheim's Annual Report) on the cell surface and synergized with TGF ß to reverse the PMA effect.

7. Regulation of Gene Expression During TGF ß Inhibition of Cell Proliferation in a Myeloid Cell Line

TGF ß1 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 ß1 inhibits the proliferation of the murine myeloid cell line 32D. Consistent with this observation is the expression of TGF ß1 65 kD type 1 receptors in these cells. In an attempt to understand the biochemical pathways that cause inhibition of cell proliferation, we have studied gene regulation by TGF ß1 in the 32D cells. Treatment with TGF ß1 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 ß1 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 ß1. Genes whose expression is tightly coupled to DNA synthesis, such as histones, are selectively inhibited by TGF ß1. Finally, TGF ß1 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 IL 3-dependent myeloid precursor cell line (32D-123) with retroviruses containing the src or abl oncogenes, were used to study transcriptional regulation of TGF ß1 mRNA. Analysis of different TGF ß1 promoter constructs regulated by $pp60^{v\text{-}src}$ 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 meyloid cells, resulting in transcriptional activation of the TGF ß1 gene. Our results demonstrate that serum treatment increases TGF ß1 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 ß1 promoter, thereby mediating the induction of

TGF ß1 promoter, leading to the induction of TGF ß1 transcription. It was surprising that abl and src transformed 32 D cl 23 cells were 10-fold more sensitive to the inhibitory effects of TGF ß. Studies using the src-transformed cells indicated that receptors for TGF ß were expressed at higher levels on these factor-independent cell lines. In addition, upon stimulation with TGF ß the receptors disappeared rapidly from the cell surface and returned to a full complement of receptors in the src-32D at least 24 hrs before the reappearance of receptors on the parental cell line suggesting that faster receptor expression plays a role in greater TGF ß sensitivity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09254-09 LMI

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.)*
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. M. Linnekin	Staff 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) BCDP, PRI/DynCorp, Frederick, MD; (Z. Howard, 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

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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 laboratory has actively investigated the molecular mechanisms of action of several human cytokines including, IL 2, IL 3, GM-CSF, and erythropoietin (Epo). 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 are designed to facilitate the biochemical purification and subsequent molecular cloning of this associated receptor specific protein kinase. In addition, we have examined the cytoplasmic domains of the Epo receptor through mutation and functional analysis and have deduced the regions required for growth promotion and protein kinase coupling. The laboratory has molecularly cloned five new human protein kinases which are expressed in normal and leukemic tissues. One tyrosine kinase designated RLK appears to be a member of a unique family of PTK recently discovered in 1990. This kinase has two catalytic domains and contains some domain similarities to the c-fms proto-oncogene. We have assigned this gene to human chromosome 1 and have found that IFNγ upregulates the mRNA of this gene in human monocytes. The laboratory has identified a transcriptional regulatory element found within the promoter regions of the IL 1Rα 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, suggesting that certain pharmacological inhibitors can block HIV transcription at this level.

PROJECT DESCRIPTION

PERSONNEL

William L. Farrar, Jr.	Senior Investigator	CMMS, LMI, NCI
Diana M. Linnekin	Staff Fellow	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 three 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; 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; and 3) development of HIV specific ribozymes for catalytic degradation of HIV RNA.

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, Epo, 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.

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. Two of the kinases cloned, designated SLK and RLK, are quite unique. RLK is a receptor-like tyrosine kinase localized on chromosome 1 and is upregulated by IFNγ treatment of human monocytes. SLK (chromosome 14) is a serine kinase we believe to be involved in glycogen metabolism and may be associated with HERS disease. We will explore the expression of this gene in relation to this genetic disorder.

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 suggests 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, Epo, 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.

We have synthesized ribozymes and added sequences that are complementary with the RRE region of the HIV genome. The ribozyme constructs anneal to this specific region of the HIV RNA genome and catalytically degrade it. We are attempting to put this HIV specific ribozyme into a mammalian vector system to determine its affects on HIV expression in lymphocytes and monocytes.

PROPOSED COURSE

The development of in vitro assays to analyse the catalytic activity of proteins associated with cytokine receptors will be explored further in aiding our efforts to biochemically characterize and molecularly clone these proteins. We will pursue those catalytic proteins associated with the IL 2, IL 3 and Epo receptors. The next priority in our future directions includes studies which determine the feasibility of testing our anti-HIV ribozyme constructs in a variety of cell lines to examine whether the transfection of anti-HIV ribozymes will inhibit HIV expression.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09360-01 LMI

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.)*
Signal Transduction Mechanisms of Hematopoietic Growth Factors

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

PI:	D. M. Linnekin	Staff Fellow	LMI, NCI
Others:	W. L. Farrar, Jr.	Senior Investigator	LMI, NCI

COOPERATING UNITS *(if any)*
Dana-Farber Cancer Institute, Boston, MA (A. D'Andrea); Immunex Research and Development Corp., Seattle, WA (L. Park); BCDP, PRI/DynCorp, Frederick, MD (Z. Howard).

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SECTION
Cytokine Molecular Mechanisms Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) Minors
☐ (a2) Interviews

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

These projects have addressed the signal transduction mechanisms of the lymphohematopoietic cytokines IL 2, IL 3, GM-CSF and Epo. Though none of the receptors for these factors encodes a protein kinase, stimulation of appropriate target cells results in increases in phosphorylation of a common subset of proteins on either tyrosine or serine residues. To better understand signal transduction mechanisms important in the proliferative response, we have characterized both a tyrosine and serine kinase substrate representing points of convergence in the signal transduction pathway of IL 2, IL 3, GM-CSF and Epo. We have found a highly conserved serine kinase substrate to be the actin binding protein l-plastin. The factor-induced phosphotyrosylprotein has several characteristics consistent with those of a tyrosine kinase. Further, this tyrosine kinase can be found in association with the Epo receptor and is constitutively phosphorylated in cells rendered Epo-independent through either transformation with Friends spleen focus forming virus (SFFV) or transfected with gp55, the envelope glycoprotein of the SFFV. In addition, our work also suggests that this protein may be associated with other cytokine receptors and may represent a protein tyrosine kinase critical for signal transduction of a number of cytokines.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

Diana M. Linnekin	Staff Fellow	CMMS, LMI, NCI
William L. Farrar, Jr.	Senior Investigator	CMMS, LMI, NCI

OBJECTIVES

The objectives of these projects has been to: 1) identify the points of convergence in the signal transduction pathways of IL 2, IL 3, GM-CSF, and Epo through examination of protein kinases and protein kinase substrates; and 2) identification of protein tyrosine kinases associated with the Epo receptor.

MAJOR FINDINGS

To characterize serine and tyrosine protein kinases important in the signal transduction of IL 2, IL 3, GM-CSF and Epo we examined protein kinase substrates as well as kinase activity. We have identified both serine kinase and tyrosine kinase substrates. A 68 kDa protein phosphorylated on serine residues in response to IL 2, IL 3, GM-CSF and Epo was identified as the actin binding protein l-plastin. Actin binding proteins have recently been shown to be associated with and regulate receptor associated signal transducing components. The relationship of p68 to the Epo receptor is currently being investigated. A 97 kDa protein was phosphorylated on tyrosine residues in response to IL 2, IL 3, GM-CSF, and Epo. A comparison of protein kinase activation in response to each of these factors showed that IL 2, IL 3, GM-CSF and Epo stimulate autophosphorylation of a common set of protein kinases. Of particular interest was factor-induced autophosphorylation of a 97 kDa protein kinase on tyrosine residues. Further investigation showed that a 97 kDa phosphotyrosylprotein present in both lymphoid and myeloid lineages bound a tyrosine kinase substrate as well as ATP. These data demonstrate that a 97 kDa tyrosine kinase is involved in the signal transduction by multiple cytokines.

The kinetics of ligand-induced tyrosine versus serine phosophorylation suggested that tyrosine kinase activation is proximal to that of the serine kinase. These results in addition to our observations of ligand-induced tyrosine phosphorylation of the receptors for IL 2, IL 3 and Epo have suggested the association of one or more tyrosine kinases with the receptors for these cytokines. Indeed, we have found an Epo-induced increase in tyrosine kinase activity in the Epo receptor complex. We have identified a 97 kDa protein in the Epo receptor complex which is phosphorylated on tyrosine residues and binds ATP. Further, this protein is constitutively phosphorylated in Epo receptor complexes isolated from cells which have been transfected with either the Friends spleen focus forming virus (SFFV) or gp55, the envelope glycoprotein shown to interact with the Epo receptor. These

results suggest that gp55, the product of the SFFV env gene, activates the Epo receptor through stimulation of a tyrosine kinase normally regulated by receptor-ligand interaction. An exciting implication of these findings is the involvement of this 97 kDa tyrosine kinase in the pathogenesis of the erythroleukemia resulting from SFFV infection.

PROPOSED COURSE

Future work will be directed at determining if the 97 kDa tyrosine kinase in the Epo receptor complex is associated with other cytokine receptors.

PUBLICATIONS

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SUMMARY REPORT

LABORATORY OF EXPERIMENTAL IMMUNOLOGY

October 1, 1990 through September 30, 1991

INTRODUCTION

Dr. John R. Ortaldo is the Chief of the Laboratory of Experimental Immunology (LEI) in the Biological Response Modifiers Program (BRMP), Division of Cancer Treatment (DCT). The LEI is composed of three sections: Leukocyte Cell Biology Section (LCBS), Dr. John R. Ortaldo, Acting Head; the Cellular and Molecular Immunology Section (CMIS), Dr. Howard A. Young, Head; and the Experimental Therapeutics Section (ETS), Dr. Robert H. Wiltrout, Head.

The LEI conducts studies on biological response modification and the application of these studies to the therapy of cancer. In-depth studies are performed on cell-mediated immune effector mechanisms, molecular biology of lymphokine gene structure and expression, lymphokine biological activities, monoclonal antibodies (MAb), growth factors, and other host responses that may be useful for cancer treatment. Selected biological response modifiers (BRMs) are studied for their effects on the immune system and other aspects of host responses, with a particular focus on the therapeutic implications of such effects. Based on such information, protocols for therapy of tumors in experimental animals and in cancer patients are developed and studies are performed to evaluate the therapeutic efficacy of selected BRMs.

LEUKOCYTE CELL BIOLOGY SECTION

The Leukocyte Cell Biology Section (LCBS) (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 LCBS conducts studies on biological response modification at the cellular and molecular levels. Specific areas of investigation include cell-mediated effector mechanisms, cytokines, MAb and growth factors. A major focus of the LCBS is the study of natural antitumor effector mechanisms and their regulation. Natural killer (NK) cells and killer (K) cells, which mediate antibody-dependent cellular cytotoxicity (ADCC), have been morphologically characterized as $CD3^-$ large granular lymphocytes (LGL) that mediate non-MHC-restricted lysis of targets. In addition to studying their cytolytic capacity, the LEI also investigates the non-cytolytic immunoregulatory functions of these natural effector cells. Specific projects involve the study of a variety of stimuli-receptor responses with the focus on signal transduction with a special emphasis on the effect of BRMs and receptor ligand interaction on intercellular signaling events. These studies include the identification and characterization a phosphatidyl inositol kinase and phosphorylation events.

The section devotes considerable attention to the study of the recognition and post-recognition events of tumor cell lysis by $CD3^-$ 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 NK cells recognize tumor cells remains incompletely elucidated, our studies have considerably enhanced the understanding of these events.

While studying surface antigens on NK-sensitive K562 tumor target cells, we have developed a MAb that blocks the binding and lysis of $CD3^-$ LGLs. An anti-idiotypic antibody also has been developed, which appears to recognize a NK antigen receptor (NK-R) and which may facilitate the identification of that receptor. This antibody reacts with 80-100 and 150 kD proteins. 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 MAbs to these 80-100 and 150 kD molecules 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.

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) MAb. These observations contributed to the discovery of the β-chain (p75) of the IL-2 receptor, which is constitutively expressed on $CD3^-$ LGLs. Our present studies are focused on studying the regulation of $CD3^-$ LGL using antibodies to the IL-2Rβ chain. In addition, studies are proceeding to examine the signal transduction events involved both in IL-2 activation and target cell interactions.

Transforming growth factor-β (TGF-β) was shown to inhibit several IL-2Rβ-mediated events in LGL and other leukocytes, including IL-2-induced NK and LAK activities, IFN-γ gene expression and secretion, and IL-2Rα expression. TGF-β inhibited these IL-2-induced LGL functions in a dose-dependent and reversible manner. Receptor analysis indicated that: 1) TGF-β had little effect on LGL IL-2Rβ expression; and 2) TGF-β receptors were not induced by IL-2. Collectively, these data indicated that TGF-β inhibited IL-2-induced $CD3^-$ LGL functions and suggested that TGF-β inhibition occurs either at the level of specific tyrosine phosphorylation and/or IL-2-induced transcriptional control factors.

The molecular and biochemical events involved in lymphocyte-mediated cytolysis are under extensive investigation. In particular, several cytoplasmic granule proteins of CTL and NK cells, including pore-forming protein (PFP, perforin or cytolysin) and serine esterases, have been extensively studied. PFP is the

major cytolytic protein of the granules and represents an important structural component of the pore-formation and granule exocytosis models for lymphocyte-mediated cytotoxicity. Recently, we have examined the regulation of PFP mRNA in human peripheral blood T cells and LGL. Our studies have indicated that IL-2 directly increases PFP mRNA in T cells. The $CD8^+$ T-cell subset was predominantly induced to express PFP mRNA. MAb to the anti-p75 IL-2 receptor abrogated IL-2 induction of PFP mRNA in T cells suggesting that IL-2 signaling via the p75 IL-2 receptor induces PFP gene expression in T cells. In contrast, high constitutive PFP mRNA expression was observed in $CD3^-$ LGL and these mRNA levels could not be enhanced by further stimulation. The cytotoxic potential of peripheral blood T cells and LGL induced in response to IL-2 correlated with IL-2 induced PFP mRNA levels in these cells and was consistent with PFP being one of several important molecules involved in the effector function of cytotoxic lymphocytes.

The LCBS has developed a model to further our understanding of the origin, differentiation and potential function of murine and human NK cells. In these studies, we have compared $CD3^-$ LGLs isolated from mouse liver with the $CD3^-$ dLyl thymocyte subset. Unexpectedly, studies have shown that the dLyl thymic subset can repopulate liver LGLs. Thus, the dLyl thymocyte appears to represent a population of cells that can regenerate liver LGLs as well as mature T cells. These studies are the first to link $CD3^-$ LGLs with a specific T-lineage progenitor. Recently, we have generated a rat MAb (4D11) that recognizes a novel 88 kD antigen (LGL-1) on the surface of LGLs isolated from mouse liver or spleen. Immunofluorescence studies indicate that this antigen is expressed on the LGLs of all strains of mice but is not detected on thymocytes, T- or B-lymphocytes or other hematopoietic populations. Further studies to examine the contribution of the LGL-1 lymphocyte subset to LAK or augmented NK activity have indicated that mouse NK cells can be divided into two major subsets, $NK1.1^+/LGL\text{-}1^+$, that mediate the majority of fresh NK activity, and $NK1.1^+/LGL\text{-}1^-$, that generate most LAK activity.

CELLULAR AND MOLECULAR IMMUNOLOGY SECTION

The Cellular and Molecular Immunology Section (CMIS; Dr. Howard Young, Head) studies the control of gene expression during the development and maturation 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 BRM gene expression is regulated during 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 through the control of specific gene expression. The specific aims of the CMIS are: 1) to study human and murine cell-mediated immunity, with emphasis on T-cell and LGL 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. Current work has focused on identifying those regions of DNA that 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, contains 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 now identified specific nucleotide sequences that interact with DNA binding proteins for each of these regions and are currently characterizing these proteins. We have also identified a human LGL-like cell line and a human B-cell line that 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. In addition, although B cells have never been reported to express IFN-γ, two B-cell lines express this protein in response to protein kinase C activators and IL-2. Current studies are focused on identifying the biochemical signaling events that contribute to the aberrant IFN-γ gene expression in these cell lines and determining if alterations in specific DNA binding proteins have occurred 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). Efforts within the CMIS have focused on a molecular analysis of the effects of FAA in vitro in order to elucidate the mechanisms by which it enhances cytokine gene expression. Efforts will be concentrated on identifying the biochemical pathways involved in this gene induction and those regulatory regions of the specific cytokine genes that are required for enhanced gene expression in response to FAA.

The CMIS, in collaboration with Dr. John Ortaldo, has also begun studies on a unique gene that functions as a receptor on NK cells (LGL) for attachment to their tumor target. This gene is located on chromosome 3, codes for a mRNA of 7-8Kb and is expressed only in T cells and LGLs. The gene contains a unique structure in the 5' extracellular region that suggests it may have a cis-trans isomerase activity. Currently studies are focused on defining conditions that alter mRNA expression, introducing cDNA expression vectors into lymphocyte cell lines in order to evaluate structure-function relationships and determining if related genes are present in lower eukaryotes.

The CMIS has also been investigating the use of cancer chemotherapeutic drugs and cytokines on the treatment of multiple myeloma. Utilizing a number of unique human myeloma cell lines, data has been generated indicating that different agents such as IL-6, retinoic acid, and phorbol esters can induce differentiation and/or growth arrest in a number of these cell lines. Current studies are focused on determining if this growth arrest can be correlated with an inhibition of specific proto-oncogene expression. In addition, nude mouse models utilizing the human tumor cell lines are being established in order to determine if the in vitro results can be correlated with in vivo tumor models.

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 combined modality approaches to cancer treatment. One aspect of these studies is to use cytokines in combination with chemotherapeutic drugs. In one such approach, performed in collaboration with Dr. Francis Ruscetti (LMI, BRMP), 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 rTGF-β1 have been shown to have potent regulatory effects on leukocytes and bone marrow-stem cells. A single intra-arterial (ia) injection, as well as the administration of multiple doses of TGF-β1 via the ip or iv routes has profound inhibitory effects on the proliferation of myeloid progenitor cells. IL-1 also has myeloproliferative and chemoprotective effects. The daily (5-7 days) administration of rIL-1α ($\geq$ 10,000 U/day) protects 70-100% of mice from acutely toxic doses of several chemotherapeutic drugs. Recently, rhIL-7 has been reported to affect the growth and function of both mature and immature T cells, as well as the differentiation pattern of myeloid progenitors. Based on these preliminary studies, we have undertaken a detailed analysis of the hematoimmunological effects of rhIL-7 in mice. The results of these studies have shown that the repeated administration of rhIL-7 to mice for 4 to 7 days induced a profound decrease (90%) in the number of CFU-c and CFU-GEMM that could be cultured from bone marrow cells and a 5- to 10-fold increase in splenic CFU-c and CFU-GEMM. Current studies are focused on 1) the cellular and molecular mechanisms for the hematological and/or chemoprotective effects of rTGF-β1, rIL-1α, and rIL-7 and 2) further investigation into improved therapeutic efficacy of intensified chemotherapy.

In other studies, various combinations of BRM or cytokines are being investigated for increased antitumor efficacy. In collaboration with Drs. Howard Young (CMIS, LEI) and Kristin Komschlies (Biological Carcinogenesis and Development Program, PRI/DynCorp), we have found that the investigational drug 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. This gene induction appears to be direct since FAA induces cytokine genes in vitro. FAA preferentially induces the gene for IFN-γ in $CD8^+$ T cells, while the gene for IFN-γ is preferentially induced in B cells. The TNF-α gene is induced in all subsets studied. FAA appears to be inactive for the direct stimulation of human leukocytes in vitro which may at least partially explain its lack of activity in human clinical trials. Similar results have been obtained with a series of xanthanone (XAA) derivatives which are structurally related to the flavonoids. Studies remain in progress to find an FAA or XAA analog that will stimulate human leukocytes.

Because of the apparent critical role of T lymphocytes in tumor rejection, studies are also in progress to evaluate the biological and antitumor effects of recombinant IL-7 (rIL-7). rIL-7 has been shown to stimulate some antigen-dependent T cell functions as well as induce LAK activity in vitro. We have demonstrated that the administration of rIL-7 to mice induces a pronounced systemic leukocytosis consisting of mostly pre-B cells with a considerable increase also in $CD8^+$ lymphocytes. Further, rIL-7 is able to reduce by 50-90% the numbers of pulmonary metastases in mice bearing murine renal cancer or MCA-38 colon carcinoma. Studies are continuing to determine whether IL-7 will be useful in inducing tumor specific T lymphocytes.

In another major research effort of the ETS, Dr. John Pearson and Dr. William Fogler have been developing CIT approaches for the intracavitary treatment of human colon (Ht-29) and ovarian (OVCAR-3) xenografted into nude mice. A human colon cell line ($Ht\text{-}29^{mdr1}$) made multidrug resistant by infection with an MDR1 retrovirus was used to study reversal of drug resistance in vivo by the anti-P-glycoprotein antibody, MRK-16. Pretreatment of $Ht\text{-}29^{mdr1}$ cells with MRK-16 MAb in vitro resulted in partial restoration of vincristine sensitivity. Similarly, the treatment of mice bearing the $Ht\text{-}29^{mdr1}$ tumor with MRK-16, before vincristine therapy, reversed resistance to the drug. In another model, the repeated intracavitary administration of 454A12 MAb-rRTA to mice bearing OVCAR-3 resulted in a significant increase in MST when compared to the control group. Co-injection of rhIFN-α and the immunotoxin resulted in 70% long-term survival as compared to only 10% survival of mice given the immunotoxin alone. Multiple treatments of 454A12 MAb-rRTA following cytoreductive therapy, at a time of minimal tumor burden, significantly increased the MST as compared to the drug treated group alone.

The ETS is also investigating the mechanisms by which BRMs augment natural immunity in vivo. Dr. Anne Pilaro has observed a BRM-induced recruitment of cells with NK activity to the liver and spleen. A modified Boyden chamber assay has been used to study the chemotaxis of NK cells in vitro. Recombinant human (rh) IL-2, rhIL-6 and rhTNF-α, but not rhIL-1α, rhIL-8, recombinant rat IFN-γ and rhTNF-β, induced directed migration of purified rat NK cells in a dose- and time-dependent fashion. Migration of NK and other cells into peripheral organs also involves adhesion and penetration through capillary endothelial barriers. Treatment of the murine vascular endothelial cell line eEND-2 with rmTNF-α or rhIL-1 resulted in 4-7 fold or 2-4 fold increases, respectively, in the adhesion of splenic NK cells, while rhIL-6, rhIL-7 or rmIFN-γ had no effect on NK cell adhesion. Preliminary studies suggest that modulation of surface adhesion molecules on eEND-2 cells by rmTNF-α is responsible for the enhanced effects. Future studies will focus on the nature of these molecules and their counter-ligands present on NK cells.

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, PRI/DynCorp). In these studies, we have purified a protein from the granules of the rat RNK leukemia cell line that is cytostatic to a variety of tumor cells. This protein shows no species specificity since certain tumor cell lines of mouse, rat, and human origin were equally sensitive to its growth inhibitory effects. Treatment of sensitive cells resulted in a rounding of the cells followed by homotypic aggregation into large aggregates. The

granule protein was distinct from cytolysin, BLT-esterase or leukolexin. It had a molecular weight of 29-31kD, bound strongly to heparin, was inactivated by heating at 70°C for 5 min or reduction, but was stable to trypsin treatment. Using molecular sieve chromatography, heparin agarose chromatography and reverse phase HPLC this protein was purified to homogeneity. The first 33 amino acids of the N-terminal amino acid sequence showed complete identity to the sequence predicted from a rat serine protease gene recently cloned and designated RNKP-1. Therefore we have purified a novel serine protease and demonstrated that it has potent effects on the growth and morphology of certain tumor cells. Other serine proteases that were structurally related and have substantial homology with RNKP-1 at the amino acid level did not show similar growth inhibitory properties on the tumor target cells we used. A serine protease inhibitor partially blocked both the morphological changes and growth inhibition produced by the granule cytostatic factor, demonstrating that the enzymatic activity of this protein was responsible for the observed biological effects.

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 09247-11 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.)*
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
	H. Young	Head, CMIS	LEI, NCI
	M. Smyth	Guest Researcher	LEI, NCI

COOPERATING UNITS *(if any)*
Immunology Branch, DCBD, NCI, Bethesda, MD (P. Henkart); BCDP, PRI/DynCorp, NCI-FCRDC (J. Rossio); and Dept. of Immunology, Juntendo Univ. School of Medicine, Tokyo, Japan (H. Yagita and K. Okumura)

LAB/BRANCH
Laboratory of Experimental Immunology

SECTION
Leukocyte Cell Biology 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. Previously, a monoclonal antibody (MAb) was developed against NK target antigens on K562 cells and MAb blocked LGL binding and lysis. We also developed an anti-idiotypic antibody (anti-ID) against this MAb anticipating that it might recognize the NK receptor and aid in its identification. This anti-ID antibody is reactive with an effector cell protein and blocks LGLs binding and target cell lysis. Utilizing this anti-ID, an expression library from CD3- LGL was screened and specific cDNA clones were isolated. The cDNA coded for a unique 150 kDa protein that consisted of several distinct structural domains. The NH2-terminal domain was 50% homologous to cyclophilin, a cyclosporin binding protein. Based on the predicted amino acid sequence from this cDNA an anti-peptide (anti-p104) antisera was prepared to further examine the structure function relationship of this novel surface moiety involved in NK lytic function.

The molecular and biochemical events involved in lymphocyte-mediated cytolysis are under extensive investigation. In particular, several cytoplasmic granule proteins of CTL and NK cells, including pore-forming protein (PFP, perforin or cytolysin) and serine esterases, have been extensively studied. PFP is the major cytolytic protein of the granules and represents an important structural component of the pore-formation and granule exocytosis models for lymphocyte-mediated cytotoxicity. Recently, we have examined the regulation of PFP mRNA in human peripheral blood T cells and LGL.

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.	ETS, LEI, NCI
Howard Young	Head	CMIS, LEI, NCI
Mark Smyth	Guest Researcher	LDS, 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; and

4. To study the nature and mechanism of cytotoxicity by LGL and to attempt to isolate and characterize soluble cytotoxic factors.

RESULTS

A. Recognition and Triggering of NK Lysis.

We have developed an IgM MAb to K562 tumor cell membrane glycoprotein, designated MAb #36. MAb #36 reacts with surface glycoprotein(s) on K562 and other NK-susceptible target cells, inhibiting binding of $CD3^-$ LGLs to target cells. It was reasoned that if this MAb reacts with a target cell molecule recognized by $CD3^-$ LGLs, then the antibody VDJ region might structurally resemble the proposed NK receptor [pNK-R]. Therefore, an anti-idiotype antibody (anti-ID) to MAb #36 might also identify the pNK-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 a pNK-R by anti-ID and have lead us to propose that the structure with which the anti-ID reacted is indeed a pNK-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 150 kD proteins. Under reducing conditions, the molecule did not separate into different chains as does the TcR, suggesting that the pNK-R is not a heterodimer like the TcR.

Based on the above results, the anti-ID was utilized in screening a human LGL cDNA expression library. A cDNA clone representing the putative pNK-R coding region was isolated. 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 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.

In an attempt to further study the molecule involved in target recognition by $CD3^-$ LGL, we have generated rabbit anti-peptide (anti-p104) antisera directed against specific amino acid sequences of our pNKR moiety. The anti-peptide (anti-p104) antisera corresponded to a portion of the ID binding region exhibited similar functional and biochemical reactivities as the anti-ID antisera. The data indicate that this novel cell surface moiety is selectively involved in both recognition and triggering of NK lytic function.

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 MAbs (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 MAbs had a reduced molecular weight of approximately 12,000 kD by immunoaffinity column purification. The kinetics of RNK-NKCF lysis of YAC-1

cells are identical to NK cell lysis, with maximal lysis seen at 4 hr thus diminishing earlier objections that NKCF could not be the lytic factor that mediates NK cell lysis due to its slow rate of killing. 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 has 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.

C. Regulation of Cytotoxic Proteins.

The mechanism by which cytotoxic T lymphocytes (CTL) rapidly lyse target cells is currently poorly understood, but T cell receptor-mediated release of granule associated proteins has been implicated in the lytic process. One of the major components found in the granules is pore-forming protein (PFP) (perforin or cytolysin), a C-9 like protein, that in the presence of $Ca2^{+}$ polymerizes to form transmembrane channels in target cells. Resting CTL exhibit no cytolytic activity contain little or no PFP. However, synthesis of PFP and demonstrated cytotoxic activity can be induced in human peripheral blood mononuclear cells (PBMC). Our studies have analyzed PFP mRNA expression in resting and stimulated human peripheral blood $CD3^{-}$ LGL, $CD3^{+}$ T cells, and their $CD4^{+}$ or $CD8^{+}$ subsets. The data indicate that IL-2 directly increases PFP mRNA in T cells in 2-4 hr, with peak levels occurring after 6 hr. The $CD8^{+}$ T cell subset was predominantly induced to express PFP mRNA. MAb to the anti-p75 IL-2 receptor abrogated IL-2 induction of PFP mRNA in T cells suggesting that IL-2 signaling via the p75 IL-2 receptor induces PFP gene expression in T cells. In contrast, high constitutive PFP mRNA expression was observed in $CD3^{-}$ LGL and these mRNA levels could not be enhanced by further stimulation with IL-2. IL-6 and suboptimal doses of IL-2 (10 U/ml) were found to co-stimulate PFP mRNA expression and cytotoxic potential in resting human peripheral blood T cells, while IL-6 or low dose IL-2 alone had no effect. The induction of T-cell PFP mRNA by IL-2/IL-6 was extremely rapid, and increases in both PFP mRNA expression and cytotoxic potential were IL-6 dose-dependent. The co-stimulatory effect of IL-6 did not appear to involve the IL-2/IL-2 receptor (IL-2R) pathway in as much as IL-6 did not induce IL-2 production or detectably increase IL-2R surface expression in T cells. These findings, in addition to the rapid induction of PFP mRNA by IL-2/IL-6, suggested that IL-6 can directly and independently provide an additional signal to augment the differentiation of CTL. In contrast to the results observed in T cells, IL-6 and IL-2 could enhance $CD3^{-}$ large granular lymphocyte (LGL) NK activity, but IL-6 either alone or in combination with IL-2 had no effect on constitutive PFP mRNA expression in resting LGL. The cytotoxic potential of peripheral blood T cells and LGL induced in response to IL-2 correlated with IL-2 induced PFP mRNA levels in these cells and was consistent with PFP being one of several important molecules involved in the effector function of cytotoxic lymphocytes.

PUBLICATIONS

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Smyth MJ, Ortaldo JR. Comparison of the effect of interleukin-2 and interleukin-6 on the lytic activity of purified human peripheral blood large granular lymphocytes. J Immunol 1991;146:1380-4.

Smyth MJ, Ortaldo JR, Shinkai Y, Yagita H, Nakati M, Okumura K, Young HA. Regulation of pore-forming protein gene expression in human peripheral blood leukocytes. In: Oppenheim JJ, Powanda MC, Kluger MJ, Dinarello CA, eds. Molecular and cellular biology of cytokines. New York: Wiley-Liss Inc, 1990; 529-34.

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Winkler-Pickett RT, Young HA, Kuta A, Ortaldo JR. Analysis of rat natural killer cytotoxic factor (NKCF): mechanism of action and relationship to other cytotoxic/cytostatic factors. Cell Immunol 1991;135:42-54.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09256-09 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.)*

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
	H. Young	Head, CMIS	LEI, NCI
	J. O'Shea	Expert	LEI, NCI
	D. McVicar	IRTA	LEI, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, NCI-FCRDC (J. Rossio); LMI, BRMP (Cytokine Mechanisms Section and Immunobiology Section); Tohoku Univ. School of Medicine, Sendai, Japan (K. Sugamura).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Leukocyte Cell Biology 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 and to generate killer cells against NK-insensitive targets and secrete IFN-γ by LGL. In addition to LGL activity being regulated by a variety of cytokines, LGLs have been shown to produce a variety of lymphokines (IL-1, IFN, CSF, BCGF). A project is being conducted to investigate IFN gene expression and regulation in highly purified human LGLs and T cells. IL-2 treatment of freshly isolated human LGLs rapidly induces IFN-γ mRNA and protein is secreted in the culture medium. Because of this one signal activation of cytokine genes, the CD3- LGL represents an excellent cell type to study signal transduction leading to both modulations of cytotoxicity and gene transcription, since it represents a cell type "poised" for activation and capable of responding to a single stimulus. Present studies involve examining agents that modulate signal transduction of IL-2 and IFN mediated events, with an emphasis on understanding the mechanism of action of biologicals and defining the specific surface receptors involved.

PROJECT DESCRIPTION

PERSONNEL

John R. Ortaldo	Chief	LEI, NCI
Joyce Frey-Vasconcells	IRTA	LDS, LEI, NCI
Anna Mason	Microbiologist	LDS, LEI, NCI
William E. Bere	Bio. Lab. Tech.	LDS, LEI, NCI
John Wine	Bio. Lab. Tech.	ETS, LEI, NCI
Howard Young	Head,	CMIS, LEI, NCI
John O'Shea	Expert	LDS, LEI, NCI
Daniel McVicar	IRTA	LDS, 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; and

3. To study the signal transduction capacities of NK cells in comparison to other lymphocytes.

RESULTS

Regulation of NK Activity

A. Activated Killers.

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. Recent studies have shown that activated NK cells may be involved in selected pathogenic states, e.g., destruction of pancreatic islet cells. In addition to their cytolytic activities, studies continue to demonstrate that $CD3^-$ LGL are important regulatory cells. Our recent studies have examined the ability of IL-2 to regulate IL-1 α/β mRNA and protein. In addition, studies have also shown that NK cells produce IL-8.

B. Murine NK Cell Studies.

Biochemical characterization of the murine NK cell associated LGL-1 antigen was performed by immunoprecipitation of surface-labeled (^{125}I) cell membranes with the 4D11 MAb. This precipitated material was analyzed on SDS-PAGE gels under reducing and nonreducing conditions and found to consist of an 87 kDa and ~42 kDa dimer. The 4D11 antibody recognizes a novel cell surface antigen, and

expression is highly correlated with the presence of LGL in all strains of mice. Studies indicate that although the antigen recognized by 4D11 (LGL-1) is not expressed on LAK precursors or effectors, it divides the NK cell population defined by NK1.1 into two subsets. Recent studies have demonstrated that LGL-1 may be a triggering surface ligand based on its ability to mediate reverse ADCC. Present studies involve attempts to clone this novel mouse receptor.

C. 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) as a result of high level expression of the β-chain receptor for IL-2. In addition, our studies have shown that monocytes and macrophages express functional receptors for IL-2 and the IL-2Rβ is the prominent signaling receptor on these leukocytes.

MAbs CD8, OKT9, OKT10, CD2, CD16, CD11, and CD56, which are specific probes for analyzing leukocyte surface molecules, were used to study the potential role of these molecules in the regulation of LGL functions. Our results with the CD2 MAb and the identification of additional CD2 epitopes led us to further examine their ability to regulate NK and LAK cytotoxicity and IFN-γ production. Although these anti-CD2s produced an approximately two-fold increase in basal LAK activity, their ability to increase and induce IFN-γ production was of considerable interest. All of the anti-CD2s except 9.6 resulted in a small increase in IFN-γ production. Combinations of CD11a [9.6] and anti-GT2 resulted in an approximately four-fold increase in basal IFN-γ units. The conclusion drawn from these data is that MAbs against the CD2 molecule are important in regulating LGL functions. Since the natural ligand for CD2 is cell surface LFA-3, this ligand interaction delivers a signal to the cell that regulates cell functions.

TGFβ was shown to inhibit several IL-2Rβ-mediated events in LGL and other leukocytes, including IL-2-induced NK and LAK activities, IFN-γ gene expression and secretion, and IL-2Rα expression. TGFβ inhibited these IL-2-induced LGL functions in a dose-dependent and reversible manner. Receptor analysis indicated that: 1) TGFβ had little effect on LGL IL-2Rβ expression; and 2) TGFβ receptors were not induced by IL-2. Studies were performed to examine binding and internalization of radiolabeled IL-2. These experiments demonstrated that the rapid binding and internalization of ^{125}I-IL-2 were not altered in $CD3^-$ LGL pretreated with TGFβ. These internalization studies indicated that the TGFβ inhibition represented post receptor-binding events in NK cells. Further studies were initiated to examine signaling events in $CD3^-$ LGL. When IL-2-induced tyrosine phosphorylation events were examined, significant inhibition was seen of selected phosphoproteins in TGFβ pretreated cells. In addition, the ability of TGFβ to also inhibit IL-2 induction of LGL IL-2Rα and IFN-γ mRNA expression was consistent with the hypothesis that post-transcriptional mechanisms were unlikely to be effected by TGFβ. Collectively, these data indicated that TGFβ inhibited IL-2-induced $CD3^-$ LGL functions and suggested that TGFβ inhibition occurs either at the level of specific tyrosine phosphorylation and/or IL-2-induced transcriptional control factors.

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 previously 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. We have continued these studies examining the tyrosine phosphorylation events in BRM activated LGL and how agents that inhibit cellular functions modulate various signaling events. Recently, we have emphasized tyrosine phosphorylation events. Our present studies are examining the activation of CD16 [FcRγIII] on NK cells, a receptor that is closely linked to tyrosine kinases [PTK] through zeta [ζ] chain for its functional activities.

Overall, our studies continue to involve the intracellular regulation of NK activity by fresh and cultured LGLs. These studies will provide insights into the maintenance of a biological function and help elucidate 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 were the case, our results indicate that PKC and PTK activities are 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. Transcription of other genes, phosphorylation of membrane and/or intracellular proteins, and metabolic activities may be equally affected. It should be possible to more precisely define the specific steps in NK activity and thereby understand the regulation of these functions at the biochemical level.

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Espinoza-Delgado I, Ortaldo JR, Winkler-Pickett R, Sugamura K, Varesio L, Longo D L. Expression and role of p75 interleukin 2 receptor on human monocytes. J Exp Med 1990;171:1821-6.

Galli M-C, Smyth MJ, Young HA, Reynolds CW, Ortaldo JR. Differential regulation of interleukin-1 gene expression in human $CD3^-$ large granular lymphocytes. Cell Immunol 1990;131:184-90.

Mason A, Bernard A, Smyth MJ, Ortaldo JR. Role of CD2 in regulation of $CD3^-$ LGL function. Eur Cytokine Net 1991;2:31-7.

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Ortaldo JR, Mason AT, O'Shea JJ, Smyth MJ, Falk LA, Kennedy ICS, Longo DL, Ruscetti FW. Mechanistic studies of transforming growth factor-β inhibition of IL-2-dependent activation of CD3$^-$ large granular lymphocyte functions: Regulation of IL2Rβ [p75] signal transduction. J Immunol 1991;146:3791-8.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09349-02 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.)*: 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. J. O'Shea	Expert	LEI, NCI
Others:	I.L.C. Kennedy	Medical Staff Fellow	LEI, NCI
	D. Eicher	Bio Tech Fellow	LEI, NCI
	D. McVicar	IRTA	LEI, NCI
	J. R. Ortaldo	Chief	LEI, NCI

COOPERATING UNITS *(if any)*: BCDP, PRI/DynCorp (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 Cell Biology 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.)*

The earliest demonstrable biochemical event that occurs as a consequence of antigen recognition by the T-cell antigen receptor (TCR) is activation of a non-receptor protein tyrosine kinase (PTK). The TCR as well as the accessory molecules CD4 and CD8 are physically associated with src family PTKs, the fyn and lck encoded kinases, respectively. We have developed a model system to elucidate the function of src family PTK in T cell function by expressing prototypic PTKs in an antigen specific T-cell hybridoma. We demonstrated that expression of v-src results in constitutive interleukin 2 (IL-2) production which is inhibitable by cyclosporin A. These cells have constitutively high nuclear levels of the transcription factor NFκB and other DNA binding proteins. The induction of NFκB is independent of protein kinase C. Other genes are induced by v-src including the IL-2Rα chain and the HTLV1-LTR. Additionally, we observed that v-src expressing T cells have marked potentiation of TCR mediated calcium mobilization which appears to be independent of phosphoinositol levels.

We have demonstrated that the NK cell Fc receptor, FcγRIIIA, is a multimeric receptor complex comprising a ligand binding molecule associated with the ζ chain and Fcεγ chains and is coupled to a PTK. Like the TCR, the ζ chain was phosphorylated upon perturbation of FcγRIIIA. We showed that pharmacologically blocking PTK activity abrogated FcR dependent calcium mobilization and induction of the IL-Rα and IFN-γ genes. We found that PTK inhibition also blocked cytotoxicity mediated by FcγRIIIA or antibody dependent cellular cytotoxicity (ADCC). We also found that PTK inhibition blocks other modes of cytotoxicity including NK mediated cytotoxicity and LAK.

Studies are underway to elucidate the structure/function relationships among src family PTK and how they influence receptor mediated signaling and gene induction.

PROJECT DESCRIPTION

PERSONNEL

John J. O'Shea	Expert	LDS, LEI, NCI
Ian L.C. Kennedy	Medical Staff Fellow	LDS, LEI, NCI
Donald Eicher	Biotechnology Fellow	LDS, LEI, NCI
Daniel McVicar	IRTA	LDS, LEI, NCI
John R. Ortaldo	Chief	LEI, NCI

OBJECTIVES

The overall goal of our studies is the elucidation of the biochemical steps involved in receptor-mediated signaling in T cells and NK cells. Specifically we have focused on T-cell receptor (TCR) and Fc receptor (FcR) mediated signaling and the role of protein tyrosine kinases (PTK). We have developed a model system to investigate the role PTK in T-cell activation by expressing prototypic PTKs such as the v-src encoded PTK and the epidermal growth factor receptor (EGFR). Using these models, we hope to better understand how src family PTKs couple the TCR to second messenger systems such as phosphoinositide (PI) turnover and calcium mobilization. Alternatively, we hope to delineate effects of this class of enzymes that are independent of PI metabolism. We hope to better understand structure/function relationships among src family PTKs and how the different family members exert these effects. Additionally, using our model systems we hope to better understand substrates that are unique to TCR mediated signaling and different src family PTK as compared to those utilized by growth factor receptors. Our goal also is to compare and contrast TCR-mediated signaling with Fc receptor (FcR) mediated signaling and ultimately compare these modes of signaling with how putative receptors involved in non-MHC restricted cytotoxicity trigger the cytolytic machinery. We would also like to characterize how the activation of PTKs translates to regulation of genes of relevance to the immune response such as cytokine genes, cytokine receptor genes and viruses such as HIV and HTLV1.

RESULTS

Activation of PTKs is one of the initial steps in TCR mediated signaling and both the TCR and the accessory molecules CD4 and CD8 are physically associated with a src-family PTK (fyn and lck encoded kinases, respectively). To better understand the function of this class of enzymes, we expressed v-src in a T cell hybridoma to determine the functional consequences. We observed that this results in constitutive low level interleukin 2 (IL-2) production that is inhibitable by Cyclosporin A. Expression of v-src also induces other genes which are important to the immune response such as the IL-2 receptor gene as well as inducing the LTRs of viruses such as HIV and HTLV1. We are delineating the transcription factors induced by v-src that may be responsible for these effects. We have determined that nuclear expression of NFκB is upregulated as a consequence of v-src expression and appears to be independent

of protein kinase C. We are presently determining if both the lck and fyn encoded PTKs have similar or disparate effects on these genes.

We also sought to determine the effect of v-src expression on TCR-mediated signaling. We found that v-src expressing cells had markedly enhanced calcium mobilization induced by perturbation of the TCR; intracellular calcium concentrations exceeded 1 μM. Surprisingly, phosphoinositol levels did not correlate with this enhanced calcium response and this effect on Ca^{2+} flux may therefore be independent of the PI pathway. We are investigating phospholipase Cγ phosphorylation nonetheless to determine if this is augmented in v-src expressing cells. We are seeking to determine if other src family PTKs have similar effects on TCR mediated signaling.

As an extension of our model system, we expressed a very different PTK in T cells, the EGFR, to determine the consequences of activation of this class of PTK in T-cell function and several interesting findings emerged. Not unexpectedly ligand binding to the EGFR expressed in T cells resulted in the phosphorylation of a number of substrates shared with occupancy of the TCR. Importantly though there were substrates that were unique to both the EGFR and TCR. We hope to use EGFR expressing T cells therefore as a tool to delineate substrates that are important in mediating TCR mediated signals. We will incorporate our findings on substrates in v-src expressing cells as well as those in T cells expressing the fyn and lck encoded kinases for fuller analysis of substrates. Additionally, we observed that while EGF stimulation did induce calcium mobilization, it did not result IL-2 production. However, EGF stimulation with perturbation of the TCR resulted in marked enhancement in IL-2 production. Again we would like ultimately to correlate these effects with phosphorylation of particular substrates. Surprisingly one substrate that is shared by TCR occupancy and EGFR activation is the ζ chain of the TCR itself. The role of ζ chain phosphorylation in TCR-mediated signaling is presently unclear but our data raises the possibility of cross-talk between the TCR and growth factor receptors.

We have demonstrated that the Fc receptor in NK cells, FcγRIIIA, like the TCR is a multimeric receptor complex comprising a transmembrane ligand binding molecule (CD16) associated with the ζ chain and Fc$\epsilon\gamma$. We also showed the FcγRIIIA is coupled to a PTK and like the TCR, this results in ζ chain phosphorylation. We have shown that inhibition of PTK activity blocks the ability of FcγRIIIA to induce calcium mobilization and influence gene expression. Thus in all respects the structure and function of FcγRIIIA is highly analogous to the TCR. We also determined that cytotoxicity mediated by FcγRIIIA antibody dependent cellular cytotoxicity was abrogated by PTK inhibition. Surprisingly though, non-MHC dependent NK mediated cytotoxicity, activated NK and lymphokine activated killing all were inhibited. Recognition of NK susceptible targets induces calcium mobilization and our preliminary data indicates that this is sensitive to herbimycin. We will therefore compare tyrosyl substrates seen upon activation of the TCR and FcR to those seen induced by recognition of NK susceptible targets to determine those that are common and those that are unique among the disparate receptors.

PUBLICATIONS

O'Shea JJ, Ashwell JD, Bailey TL, Cross SL, Samelson LE, Klausner RD. Expression of v-src in a murine T-cell hybridoma results in constitutive T-cell receptor phosphorylation and interleukin 2 production. Proc Natl Acad Sci USA 1991;88:1741-5.

O'Shea JJ, Ortaldo JR. Killer T's, macrophages, and NK's. The New Biologist 1991;2:779-82.

O'Shea JJ, Weissman AM, Kennedy ICS, Ortaldo, JR. Engagement of the natural killer cell IgG Fc receptor results in tyrosine phosphorylation of the ζ chain. Proc Natl Acad Sci USA 1991;88:350-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09283-07 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.)*: 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	Biol Lab Tech	LEI, NCI
	D. Reynolds	Biologist	LEI, NCI
	P. Ghosh	Staff Fellow	LEI, NCI
	A. Sica	Visiting Fellow	LEI, NCI
	Y. Pang	Visiting Fellow	LEI, NCI

COOPERATING UNITS *(if any)*: Biological Carcinogenesis and Development Program, PRI/DynCorp, NCI-FCRDC (L. Dorman, L.A. Eader).

LAB/BRANCH: Laboratory of Experimental Immunology

SECTION: Cellular and Molecular Immunology Section

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

TOTAL MAN-YEARS: 4.7	PROFESSIONAL: 3.2	OTHER: 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 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. We are now identifying the DNA binding proteins which interact with the IFN-γ genomic DNA and comparing the levels of these proteins in purified lymphocyte subsets. These studies will enable us to determine if the extra cellular signaling which results in IFN-γ gene expression correlates with a modulation in the levels of specific DNA binding proteins.

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Allen Wilt	Biol Lab Tech	CMIS, LEI, NCI
Della Reynolds	Biologist	CMIS, LEI, NCI
Paritosh Ghosh	Staff Fellow	CMIS, LEI, NCI
Antonio Sica	Visiting Fellow	CMIS, LEI, NCI
Yubin Pang	Visiting Fellow	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

Based on our previous studies, we have dissected the non-coding regions of the human DNA to determine which regions enhance gene transcription in response to extracellular signals. This structural analysis of the gene has indicated that both enhancer and repressor activity can be detected within the 300 bp 5' to the TATAA box. We have now identified three putative proteins which bind to this enhancer region and at least one protein which binds to the silencer region. Additional proteins have been identified which bind to the intronic enhancer and it appears that both c-Rel and NFλB may bind to this region of DNA.

We have also identified unique B-cell lines which are capable of expressing their endogenous IFN-γ gene as both mRNA and protein. One of these cell lines can be induced to produce IFN-γ by both IL-2 and PKC activating agents while the other cell line can be induced only by PKC activating agents. The ability of IL-2 to induce gene expression can be directly correlated with the levels of the IL-2 receptor β chain which appear on the cell surface. The mechanisms responsible for this altered and unusual production of IFN-γ in B-cell lines 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 immunomodulatory molecule in immune system development. By elucidating how the defined regulatory regions are involved in the induction, altered expression and 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

Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, Wolf SF, Young D, Clark SC, Trinchieri G. Induction of interferonγ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med 1991;173:869-79.

Chrivia JC, Wedrychowicz T, Young HA, Hardy KJ. A model of human cytokine regulation based on transfection of γ interferon gene fragments directly into isolated peripheral blood T lymphocytes. J Exp Med 1990;172:661-4.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09303-05 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.)*: Studies of Human B Cell Malignancies

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

PI:	H. A. Young	Head	LEI, NCI
Other:	Y. Pang	Visiting Fellow	LEI, NCI

COOPERATING UNITS *(if any)*: Biological Carcinogenesis and Development Program, PRI/DynCorp, NCI-FCRDC (R. R. S. Kantor)

LAB/BRANCH: Laboratory of Experimental Immunology

SECTION: Cellular and Molecular Immunology Section

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

TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Human multiple myeloma is a malignancy of hematopoietic stem cell origin which manifests itself at the mature stage of B-cell development. The present studies examine biological approaches to the treatment of human myeloma. Interleukin-6 (IL-6) has been shown to be an autocrine or paracrine growth factor for myeloma cells. One human myeloma cell line, U-266, is growth inhibited by monoclonal antibodies (MAb) to IL-6 and will be used in IL-6 signaling studies and in vivo models of human myeloma in nude or scid mice. In conjunction with these studies, MAb to IL-6 and the IL-6 receptor will be used in a clinical trial of myeloma patients at the BRMP. As human myeloma cell lines have been traditionally difficult to establish, we have recently established a human myeloma line from an IgG myeloma patient. This cell line and additional myeloma cell lines will also be utilized for in vitro and in vivo studies on the effects of cytokines and chemotherapeutic drugs on myeloma growth and differentiation.

We have observed that several agents, including IFNα, retinoid acid, phorbol esters, ionomycin, and hexamethylene bisacetamide, which in other systems promote cellular differentiation, inhibit myeloma cell line growth. We are currently investigating the mechanism by which these agents act on myeloma cells and extending these findings to in vivo myeloma model systems.

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Head	CMIS, LEI, NCI
Yubin Pang	Visiting Fellow	CMIS, LEI, NCI

OBJECTIVES

The objectives of this project are 1) to investigate biological approaches to the treatment of human multiple myeloma 2) to study the role of IL-6 in the development of human multiple myeloma.

RESULTS

We have screened biological agents for their ability to inhibit the growth of human myeloma cell lines in vitro. IFNα has been previously found to inhibit myeloma cell line growth and we have now found that retinoid acid also inhibited myeloma growth and produced an enhanced effect in combination with IFNα. The phorbol esters, found to be very active in inducing cellular differentiation, also inhibited myeloma growth and when used in combination with ionomycin produced an enhanced growth inhibitory effect. As phorbol esters act by stimulation of protein kinase C, we have found a differential effect of PKC activators on myeloma cell line growth. In contrast to the effects of PMA, teleocidin, indolactin, and bryostatin do not inhibit myeloma growth. Additionally, when added in combination with PMA, bryostatin was able to antagonize the PMA growth inhibitory effect. One human myeloma cell line, HS-Sultan, underwent a striking morphological transformation when exposed to phorbol esters and this was accompanied by (i) alterations in cell surface marker expression (ii) production of lymphokines and cytokines (iii) arrest at the G2/M stage of cell cycle and (iv) a down-modulation of c-myc mRNA levels. These results suggest a potential role for agents that promote cellular differentiation in the therapy of multiple myeloma.

PUBLICATION

Kantor RRS, Giardina SL, Bartolazzi A, Townsend AJ, Myers CE, Cowan KH, Longo DL, Natali PG. Monoclonal antibodies to glutathione S-transferase π-immunohistochemical analysis of normal human tissues and cancers. Int J Cancer 1991;47:193-201.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09326-03 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.): 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, PRI/DynCorp, 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: 0.7	PROFESSIONAL: 0.2	OTHER: 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. We have now found flavone can act directly on mouse macrophage cell lines in vitro and induced specific cytokine gene expression. These results indicate that FAA acts by directly stimulating gene expression of 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 (IL-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 defined the kinetics of cytokine mRNA induction after in vivo administration of FAA. The aim of this investigation was to define the induction in cell lines, of mRNA for IFN or other cytokines following FAA administration in order to develop a model system for dtermining the mechanism of flavone action.

ACCOMPLISHMENTS

A panel of cytokine probes was employed in the in vitro screening of mouse macrophage cell lines. Both IL-6 and interferon beta mRNAs were found to be rapidly induced in a dose dependent manner by flavone. These results demonstrate that FAA can act as a potent inducer of cytokine mRNA in vitro and indicate that the mouse macrophage cell lines will be an important tool for elucidating the biochemical pathways by which flavone directly induces gene expression.

Additional studies are being undertaken to determine if derivatives of FAA can also induce cytokine gene expression in human lymphocytes in vitro as FAA by itself is inactive on human cells.

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;3:219-32.

Wiltrout RH, Hornung RL, Futami H, Back TT, Young HA, Sayers TJ. Murine renal cancer (Renca) model: background and preclinical studies. In: Debruyne FMJ ed. Recent advances on the immunotherapy of renal cell cancer. Heidelberg: Springer-Verlag, 1991;13-9.

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. Oxford: IRL Press, 1990;90-7.

Young HA, Eader L, Futami H, Ayroldi E, Varesio L, Pitha-Rowe P, Wiltrout RH. Modulation of immune system function by chemotherapeutic drugs: induction of cytokine gene expression and activation of a cytokine gene promoter activity by the investigational chemotherapeutic drug, flavone acetic acid. In: Streilein JW, Ahmad F, Bialy H, Black S, Blomberg B, Chin YH, Lopez D, Malek T, Podack ER, Rabin MB, Stein-Streilein J, Van Brunt J, Whelan WJ, eds. Proceedings of the 1990 Miami Bio/Technology Winter Symposia Advances in Gene Technology: The Molecular Biology of Immune Diseases and the Immune Response, vol. 10. Miami: 1990 Miami Bio/Technology Winter Symposia, 1990:141.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09345-02 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.)*
Molecular Studies of Cellular Cytotoxicity

PRINCIPAL INVESTIGATOR *(List other professional 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	Nakasone 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 been characterizing a new gene which appears to be a receptor for LGLs and is expressed in the cytoplasm of T cells. We have also correlated cytotoxic function with expression of the cytotoxic protein, perforin, and have identified cytokines 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	Nakasone 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, region 3p21-3p23, and is expressed only in T cells and LGLs. The gene contains a unique structure in the 5' extracellular region which suggests that it may have a cis-trans isomerase activity and bind cyclosporin A. Currently studies are focused on defining conditions which alter mRNA expression and on the effects of introducing antisense cDNA expression vectors containing 5' portions of the cDNA into appropriate lymphocyte cell lines in order to evaluate the effects of inhibiting gene expression on cytotoxic mechanisms.

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. We have now found that IL-7 can also enhance gene expression in the $CD8^+$ population but not in LGLs. However, the effects of IL-7 on perforin gene expression and CD8 cytotoxic activity is not as great as that seen with IL-2. We have also found that upon the proper conditions of activation, $CD4^+$ cells can also be induced to synthesize perforin mRNA.

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. Furthermore, we will attempt to correlate the role of perforin gene expression in the cytotoxic activity mediated by certain $CD4^+$ cell populations. Thus based on a precise understanding of NK receptor gene expression and perforin gene expression, it may be possible to design unique drugs which provide a stimulus to specific cell populations in 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 T cells or

large granular lymphocytes and enhances the functional activity of these cells.

PUBLICATIONS

Galli M-C, Smyth MJ, Young HA, Reynolds CW, Ortaldo JR. Differential regulation of interleukin-1 gene expression in human $CD3^-$ large granular lymphocytes. Cell Immunol 1990;131:184-90.

Latham PS, Lewis AM, Varesio L, Pavlakis GN, Felber BK, Ruscetti FW, Young HA. Expression of human immunodeficiency virus long terminal repeat in the human promonocyte cell line U937: effect of endotoxin and cytokines. Cell Immunol 1990;129:513-8.

Smyth MJ, Ortaldo JR, Shinkai Y-I., Yagita H, Nakata M, Okumura K, Young HA. Regulation of pore-forming protein gene expression in human peripheral blood leukocytes. In: Oppenheim JJ, Powanda MC, Kluger MJ, Dinarello CA, eds. Molecular and cellular biology of cytokines. New York: Wiley-Liss Inc, 1990;529-34.

Smyth MJ, Strobl SL, Young HA, Ortaldo JR, Ochoa AC. The regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood $CD8^+$ T lymphocytes: inhibition by transforming growth factor-β. J Immunol 1991;146:3289-97.

Winkler-Pickett RT, Young HA, Kuta A, Ortaldo JR. Analysis of rat natural killer cytotoxic factor (NKCF): mechanism of action and relationship to other cytotoxic/cytostatic factors. Cell Immunol 1991;135:42-54.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09262-09 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.)*

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
	N. Usui	Visiting Fellow	LEI, NCI
	M. MacPhee	Visiting 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, PRI/DynCorp, NCI-FCRDC (T. Sayers, K. Grzegorzewski, 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. The mechanism for this gene induction remains unclear, but is thought to be directly mediated since 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-γ 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. Interestingly, FAA appears to be inactive for the direct stimulation of human leukocytes in vitro which may at least partially explain its lack of activity in human clinical trials. Similar results have been obtained with a series of xanthanone (XAA) derivatives which are structurally related to the flavoids. These compounds are also potent cytokine inducers in mice but inactive on human cells. Studies remain in progress to find an FAA or XAA analog that will stimulate human leukocytes. Because of the apparent critical role of T lymphocytes in tumor rejection, studies are also in progress to evaluate the biological and antitumor effects of recombinant interleukin 7 (rIL-7). rIL-7 has been shown to stimulate some antigen-dependent T-cell functions as well as induce LAK activity in vitro. We have demonstrated that the administration of rIL-7 to mice induces a pronounced systemic leukocytosis consisting of mostly pre-B cells with a considerable increase also in CD8+ lymphocytes. Further, rIL-7 is able to reduce by 50-90% the numbers of pulmonary metastases in mice bearing murine renal cancer or MCA-38 colons adenocarcinoma. Finally an apparently novel tumor cytostatic factor has been purified and sequenced from lysates of rat RNK tumor lines, and its biological effects are now being evaluated.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS, LEI, NCI
Anne M. Pilaro	Staff Fellow	ETS, LEI, NCI
Noriko Usui	Visiting Fellow	ETS, LEI, NCI
Martin Macphee	Visiting Fellow	ETS, LEI, NCI
M. Eilene Gruys	Biologist	ETS, LEI, NCI
Theresa A. 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 flavonoid 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 T cell-stimulating cytokines (TNF-α, IFN-α, γ, IL-6, and IL-7) in combined modality approaches to the treatment of various mouse tumors.

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 flavonoids plus rIL-2 mediate antitumor effects against Renca; 2) studying the ability of T cell-stimulating cytokines, to mediate antitumor effects in combination with rIL-2 against mouse tumors; and 3) studies are in progress to characterize the biological functions of a recently identified factor that is cytostatic for tumor cells in vitro.

Flavone-8 acetic acid (FAA) is a flavonoid drug that augments mouse natural killer (NK) activity, induces cytokine gene expression, and synergizes with recombinant interleukin 2 (rIL-2) for the treatment of murine renal cancer (Renca). The dose-response curves for all of these FAA-induced biological effects are extremely steep and superimposable suggesting a role for FAA-induced cytokine expression in the therapeutic effects of FAA treatment to directly induce cytokine mRNA expression in total mouse splenic leukocytes and

selected leukocyte subsets. Analysis of RNA isolated from FAA-treated mouse splenic leukocytes demonstrated that treatment with ≥ 100 μg/ml FAA induced maximal expression of TNF-α, IFN-α and IFN-γ mRNA within 3 hours. Increased TNF-α gene expression was detectable by 1 hr and diminished by 6 hours. These results correlate well with the previously reported induction of cytokine mRNA genes and biological activity by FAA in vivo, and suggest that these effects are direct. Further studies performed with highly purified B cells, demonstrated that the FAA-induced expression of IFN-γ mRNA was mainly induced in the $CD8^+$ T lymphocyte subset. IFN-α mRNA was expressed largely in the B cell population, while TNF-α mRNA was induced in all leukocyte subsets tested. In contrast, FAA did not increase mRNA or increase cytokine protein secretion by human PBL under similar conditions. These results demonstrate that FAA can directly stimulate cytokine gene expression in mouse but not in human leukocytes. Therefore, these results suggest that the failure of FAA as a therapeutic agent in humans could relate to its inability to induce cytokine genes in human leukocytes. Flavonoid compounds or analogues that stimulate cytokine gene expression in human cells might be therapeutically active in cancer patients.

Derivatives of xanthanone-4-acetic acid (XAA) have also been found to have similar activity to flavone-8-acetic acid against transplantable solid tumors. Some of these compounds were compared to flavone acetic acid (FAA) in their ability to induce cytokines as well as to mediate antitumor effects against a murine renal cancer (Renca). 5-Methyl XAA and 5-chloro XAA proved to be more potent than FAA on a mg/kg basis for induction of the genes for IFN-α, IFN-γ, and TNF-α, and TNF activities in the sera of treated mice. These effects were sharply dose-dependent. On the other hand, 7-methyl XAA, which has no antitumor activity did not induce these genes. In addition, 5-methyl XAA and 5-chloro XAA, but not 7-methyl XAA synergized with recombinant human interleukin 2 (rhIL-2) for the successful treatment of Renca. Doses of the active derivatives that failed to induce cytokines also exhibited no therapeutic synergy with rhIL-2. These results suggest that at least some of the antitumor effects of these XAA derivatives are related to their ability to induce cytokines.

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.

T lymphocytes are particularly potent antitumor effector cells in general, and are involved in several of our mouse models. Cytokines that stimulate T cells should ultimately prove very useful in concert with other immunoactive cytokines and/or with chemotherapeutic drugs. Recently, rhIL-7 has been reported to affect the growth and function of both mature and immature T cells, as well as the differentiation pattern of myeloid progenitors. Based on these preliminary studies, we have undertaken a detailed analysis of the hematoimmunological effects of rhIL-7 in mice. The results of these studies have shown that the administration of daily doses of 5 and 10 μg rhIL-7 to

mice for 4 to 7 days induces a pronounced (5-10 fold) leukocytosis in the peripheral blood and spleen, and about a 3 fold increase in lymph node cellularity. This increase occurs largely because of an increase in $B220^+$ B lymphocytes. In addition there is an increase in the total number of both $CD4^+$ and $CD8^+$ T cells, with the effects on $CD8^+$ T cells predominating such that the CD4/CD8 ratio goes from about 2:1 to 0.8:1. Coincident with these phenotypic changes, an increase in baseline proliferation rate was detected. Further studies revealed that rhIL-7 administration also induced a profound decrease (90%) in the number of CFU-c and CFU-GEMM that could be cultured from bone marrow cells and a 5 to 10-fold increase in splenic CFU-c and CFU-GEMM.

Because the administration of rhIL-7 to mice caused dramatic perturbations in the frequency of hematopoietic progenitor cells, as well as alterations in lymphocyte subsets, studies were performed to determine whether rhIL-7 would have antimetastatic properties in mice. The data demonstrated that the twice daily administration of rhIL-7 at doses $\geq 5\mu g$/injection for 5-10 days appreciably inhibited the number of pre-existent experimentally induced Renca and MCA 38-induced metastases in the lungs of syngeneic mice. This effect is dose-dependent. Preliminary data obtained from experiments designed to study effects of rhIL-7 on hepatic Renca metastases suggest that rhIL-7 may enhance the antimetastatic effects of rhIL-2. Overall, these results demonstrate the rhIL-7 has antimetastatic effects against at least some rodent tumors, and suggests that a further investigation of its antimetastatic potential is warranted.

In additional studies, we have purified a protein from the granules of the rat RNK leukemia cell line that is cytostatic to a variety of tumor cells. This protein shows no species specificity since certain tumor cell lines of mouse, rat, and human origin were equally sensitive to its growth inhibitory effects. Treatment of sensitive cells resulted in a rounding of the cells followed by homotypic aggregation into large aggregates. The granule protein was distinct from cytolysin, BLT-esterase or leukolexin. It had a molecular weight of 29-31kD, bound strongly to heparin, was inactivated by heating at 70°C for 5 min or reduction, but was stable to trypsin treatment. Using molecular sieve chromatography, heparin agarose chromatography and reverse phase HPLC this protein was purified to homogeneity. The first 33 amino acids of the N-terminal amino acid sequence showed complete identity to the sequence predicted from a rat serine protease gene recently cloned and designated RNKP-1. Therefore we have purified a novel serine protease and demonstrated that it has potent effects on the growth and morphology of certain tumor cells. Other serine proteases that were structurally related and have substantial homology with RNKP-1 at the amino acid level did not show similar growth inhibitory properties on the tumor target cells we used. A serine protease inhibitor partially blocked both the morphological changes and growth inhibition produced by the granule cytostatic factor, demonstrating that the enzymatic activity of this protein was responsible for the observed biological effects.

PUBLICATIONS

Futami H, Hornung RL, Back TT, Bull R, Gruys E, Wiltrout RH. Systemic alkalinization inhibits the ability of flavone acetic acid to augment natural killer activity, induce cytokine gene expression, and synergize with interleukin 2 for the treatment of murine renal cancer. Cancer Res 1990;50:7926-31.

Futami H, Jansen R, MacPhee MJ, Keller J, McCormick K, Longo DL, Oppenheim JJ, Ruscetti FW, Wiltrout RH. Chemoprotective effects of recombinant human IL-1α in cyclophosphamide-treated normal and tumor-bearing mice: protection from acute toxicity, hematologic effects, development of late mortality and enhanced therapeutic efficacy. J Immunol 1990;145:4121-30.

Futami H, Pilaro A, Gruys ME, Wiltrout RH. Enrichment of murine LAK effector cells. In vivo localization and cytokine gene expression. Biotherapy 1990;in press.

Ortaldo JR, Wiltrout RH. Editorial: Implications of potential positive correlation between autologous tumor-cell-killing activity and prognosis of human lung cancer. J Natl Cancer Inst 1990;82:1663-5.

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 α-interferon. Cancer Res 1990;50:6379-88.

Pilaro AM, Sayers TJ, McCormick KL, Reynolds CW, Wiltrout RH. An improved in vitro assay to quantitate chemotaxis of rat peripheral blood large granular lymphocytes (LGL). J Immunol Methods 1990;135:213-24.

Pilaro AM, Sayers TJ, Wiltrout RH. Potential mechanism for the recruitment of large granular lymphocytes following treatment with biological response modifiers. In: Powanda MC, Oppenheim JJ, Klugar MJ, Dinarello CA, eds. The physiological and pathological effects of cytokines. Boston: Wiley-Liss Inc., 1990;381-6.

Wiltrout RH, Damia G, MacPhee M, Futami H, Faltynek CR, Longo DL, Ruscetti FW, Komschlies KL. Cytokine-based combined modality approaches to the treatment of murine renal cancer. In: Goldstein AL, Garaci E, eds. Proceedings of the Combination Therapies, New and Emerging Uses for Biological Response Modifiers in the Treatment of Cancer and Infectious Diseases. Washington, D.C.: Institute for Advanced Studies in Immunology and Aging, 1991;in press.

Wiltrout RH, Hornung RL, Futami H, Back TT, Young HA, Sayers TJ. Murine renal cancer (Renca) model: Background and preclinical studies. In: Debruyne FMJ, Bukowski RM, Pontes JE, deMulder PHM, eds. Immunotherapy of renal cell cancer. Heidelberg: Springer-Verlag, 1990:13-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09288-06 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.)*: Biological and Chemotherapeutic Modalities Against Human Tumors In Nude 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
	N. Usui	Visiting Fellow	LEI, NCI
	W.E. Fogler	Senior Investigator	CTEP, NCI
	S.E. Bates	Senior Clinical Investigator	DCT, NCI
	M.M. Gottesman	Head	LCB, NCI

COOPERATING UNITS *(if any)*: Biological Carcinogenesis and Development Program, PRI/DynCorp, NCI-FCRDC (T. Sayers, K. Volker and R. Kantor); Cetus Corp. (E. Groves); Gowsala Sivam (Fred Hutchinson Research Center).

LAB/BRANCH: Laboratory of Experimental Immunology

SECTION: Experimental Therapeutics Section

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

TOTAL MAN-YEARS: 2.25	PROFESSIONAL: 1.50	OTHER: .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.)*

A human colon cell line (Ht-29mdr1) made multidrug resistant by infection with an MDR1 retrovirus was used to study reversal of drug resistance in vivo by the anti-P-glycoprotein antibody, MRK-16. The 50% inhibitory concentrations (IC50) of vincristine on Ht-29par and Ht-29mdr1 cells was 2.5 and 15ng/ml, respectively. Pretreatment of Ht-29mdr1 cells with 10μg/ml MRK-16 MoAb in vitro resulted in partial restoration of vincristine sensitivity (IC50=7ng/ml). Studies showed that MSTs of mice transplanted ip with 5,000,000 Ht-29par or Ht-29mdr1 were 37 and 39 days respectively. Treatment of mice with 1mg/kg vincristine weekly for three weeks beginning 10 days post-tumor cell injection resulted in a significant increase in MST of the Ht-29par tumor bearing mice (68 days, p<.0001), but had no effect on the Ht-29mdr1 mice. However, treatment of mice bearing the Ht-29mdr1 tumor with 500μg MRK-16, before vincristine therapy, reversed resistance to the drug (MST=64 days, p<.0001). The intracavity administration of 454A12 MAB-rRTA (10μg) to mice bearing OVCAR-3 beginning 10 days post-tumor cell inoculation and then every other day for a total of 10 treatments resulted in a significant increase in MST of 89 days (p<.0001) when compared to the control group (41 days). Coinjection of 50,000 units of rhIFN-α and the IT at a similar regimen resulted in 70% long term survival when compared to only 10% survival of mice given the IT alone. The ip injection of 454A12 MAB-rRTA alone or in combination with rhIFN-α administered by an identical regimen beginning 15 days post-tumor cell injection resulted in significant (p<.0001) increases in MSTs of 72 and 75 days, respectively. No long term survivors were observed. Multiple treatments of 454A12 MAB-rRTA (10μg) following cytoreductive therapy, at a time of minimal tumor burden, exhibited a significant increase (p<.0001) in MST of 123 days when compared to the drug treated group alone (MST=76 days). Finally, the combination of the IT and rhIFN-α resulted in an enhanced therapeutic response (35% long term survivors) when compared to the IT alone (19%).

PROJECT DESCRIPTION

PERSONNEL

John W. Pearson	Microbiologist	ETS, LEI, NCI
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OBJECTIVES

The aim of this project is two-fold: (1) To develop and utilize animal models to overcome drug resistance of human tumors in vivo. The specific aims of this objective are: (a) to examine various drugs that may reverse resistance and (b) to study the use of a specific gp 170 monoclonal antibody (MRK-16) alone or coupled to cytotoxic substances to attempt treatment of drug resistant tumors and (2) to evaluate the intracavitary administration of immunotoxins (ITs) 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, (dose and regimen), to maximize the therapeutic effect of specific ITs against localized tumor growth and (b) to assess the feasibility of combination therapy (BRMs or chemotherapy) in combination with ITs to further enhance the therapeutic efficacy of ITs against peritoneal disease.

RESULTS

1. In Vitro and In Vivo Reversal of Vincristine Resistance

Over the past year, studies have been underway to investigate reversal of vincristine resistance by the anti-P-glycoprotein antibody, MRK-16, in nude mice bearing a multidrug resistant infected human colon xenograft. Following infection of the human colon tumor line (Ht-29^{mdr1}) with the MDR1 gene, flow cytometric analysis revealed the fluorescent intensity of Ht-29^{mdr1} cells for MRK-16 was more than two-fold greater than that seen with the parental, non-infected colon line (Ht-29^{par}). Furthermore, scatchard analysis studies demonstrated that the mean number of MRK-16 specific binding sites/cell was at least 15-fold greater for Ht-29^{mdr1} (408,000) as compared to the Ht-29^{par} cells. Therefore, these data indicate that infection with the MDR1 retrovirus increased the phenotypic expression of MRK-16 reactive sites on the Ht-29^{mdr1} cell line. Studies were then undertaken to determine whether MRK-16 would modulate vincristine cytotoxicity to the Ht-29^{par} and Ht-29^{mdr1} cells. In vitro sensitivity of the Ht-29^{par} and Ht-29^{mdr1} cells to vincristine was determined by the MTT assay. The IC_{50} for vincristine on Ht-29^{par} cells following 48 h of

culture was 2.5ng/ml. Under identical culture conditions, the IC_{50} of vincristine on Ht-29^{mdr1} cells was determined to be 15ng/ml. Further, the pretreatment of Ht-29^{mdr1} cells with 10μg/ml of MRK-16 resulted in a 46% decrease in the IC_{50} of vincristine (7ng/ml) as compared to control incubations. Therefore, these studies demonstrated that the increase in gp 170 expression in the Ht-29^{mdr1} cell line correlated with approximately a 6-fold increase in resistance to vincristine and thus resistance could be partially reversed by treatment with MRK-16.

The ability of MRK-16 to modulate VCR antitumor activity in vivo was studied in nude mice. Ten days after the ip injection of $5x10^6$ Ht-29^{par} or Ht-29^{mdr1} cells, mice were treated weekly for three weeks with 1 mg/kg VCR alone or in combination with 500 μg MRK-16. Under these conditions, the MST of Ht-29^{par} tumor bearing mice treated with saline or MRK-16 alone was 37 and 34 days, respectively. Treatment of Ht-29^{par} tumor bearing mice with VCR significantly increased the MST to 68 days ($p<.0001$). This MST was unaffected by the inclusion of MRK-16 in the treatment regimen (MST = 65 days). A different pattern of treatment response was observed in mice transplanted with the Ht-29^{mdr1} ascites cell line. The MST of Ht-29^{mdr1} tumor-bearing mice with VCR and MRK-16 resulted in a highly significant increase in MST compared to control or drug treated groups (64 days, $p<.0001$). Concomitant treatment of Ht-29^{mdr1} tumor-bearing mice with VCR and a control NR-LU-10 MoAb did not result in a change in the MST (42 days) as compared to mice treated with VCR alone. These data imply that the reversal of in vivo VCR resistance in Ht-29^{mdr1} cells by MRK-16 was not due to a nonspecific membrane alteration that occurred as a consequence of MoAb binding. Studies are ongoing in vivo to evaluate various other drug reversal agents such as verapamil, cyclosporin A, and quinidine sulfate. Such agents have been demonstrated to decrease cellular efflux of cytotoxic drugs and to bind to gp 170. Finally, arrangements are being made to have MRK-16 monoclonal antibody conjugated to a specific toxin, Verrucarin A, because a specific immunotoxin directed at the gp 170 may offer more efficient modulation of drug resistance by altering the resistance mechanism to other drugs as well as by directly killing these cells.

2. In Vivo Antitumor Activity of an Immunotoxin Against OVCAR-3

Recently, studies were published involving the use of a specific IT 454A12 MAB-rRTA, alone or in conjunction with recombinant human α-interferon (rhIFNα) against a human ovarian carcinoma cell, OVCAR-3, xenografted into nude mice. Cohorts of mice that received intracavitary treatment beginning 5 days post-tumor cell inoculation, a time when approximately $1x10^7$ ascites cells could be recovered from the peritoneal cavity, with 454A12 MAB-rRTA resulted in a highly increased MST when compared to the untreated control group. Furthermore, it was demonstrated that the in vivo activity of the IT was further enhanced with concomitant administration of a nontherapeutic dose of rhIFN-α characterized by an increase in the prolongation of survival as well as an increased percentage of long term survivors. Therefore, studies have been underway to further explore this modality of therapy when a greater tumor burden was evident following tumor cell inoculation. To assess the in vivo antitumor efficacy of 454A12 MAB-rRTA, mice were injected i.p. with $5x10^6$ ascites cells. Beginning 10 days post tumor cell inoculation, a time when

approximately $2.5x10^7$ ascites cells could be recovered from the peritoneal cavity, mice were treated i.p. every other day with 10μg of 454A12 MAB-rRTA alone or in combination with rhIFN-α ($5x10^4$ units). Mice that received the IT alone exhibited a significant increase ($p<.0001$) in MST of 89 days when compared with the saline control group (41 days). Treatment of mice with $5x10^4$ units of rhIFN-α and 10μg of 454A12 MAB-rRTA not only increased the life span of the treated mice but also resulted in the long term survival of 70% of the mice as compared with 10% survivors in the group that received the IT alone when the investigation was terminated at 120 days. Studies were then undertaken to see what effect this modality of therapy had against an even greater OVCAR-3 tumor burden. In this case, following the i.p. injection of $5x10^6$ ascites cells, cohorts of mice were treated with 454A12 MAB-rRTA (10μg) alone or with $5x10^4$ units of rhIFN-α at a similar regimen beginning 15 days post-tumor cell inoculation. At this time, approximately $4x10^7$ ascites cells could be recovered from the peritoneal cavity of individual mice. Under these conditions, the MST of mice treated with saline was 35 days. In contrast, mice given multiple treatments of the IT alone or in combination with rhIFN-α resulted in highly significant ($p<.0001$) increases in MSTs of 72 and 75 days respectively. However, coadministration of rhIFN-α with the IT resulted in no significant differences ($p=0.33$) in MSTs when compared to the group of mice treated with 454A12 MAB-rRTA. In addition, there were no long term survivors when the study was terminated at 120 days.

Since it was evident that the initial tumor burden was crucial for the effectiveness of IT therapy, it was of interest to see what effect this modality of therapy had alone or in combination with rhIFN-α against the OVCAR-3 tumor when administered at a time of minimal tumor burden and after cytoreductive therapy. In this case mice were treated with cytoxan (200mg/kg) and cis-platin (5mg/kg) beginning 20 days post tumor cell inoculation, a time when approximately $4.5x10^7$ ascites cells could be recovered from the peritoneal cavity as well as the existence of 6x10mm peritoneal solid tumor implants. One week later (day 27), mice received a second course of cytoxan (100mg/kg) and cis-platin (2.5mg/kg). Forty days post tumor cell inoculation, a time when one could recover approximately $2.5x10^6$ ascites cells from the peritoneal cavity in the absence of gross peritoneal implants, IT therapy alone or in combination with rhIFN-α was initiated every other day for a total of 10 treatments. One observed a significant increase in the MST (from 37 to 76 days) in the drug treated group when compared to the untreated mice ($p<.0001$) was observed. Furthermore, the administration of $5x10^4$ units of rhIFN-α alone following cytoreductive therapy caused a further increase in MST ($p<.0001$) to 89 days when compared to the group treated with cytoxan and cis-platin (76 days). The therapeutic modality of multiple treatments of 10μg of 454A12 MAB-rRTA following chemotherapy significantly prolonged survival time ($p<.0001$) of mice (MST=123 days) when compared to the drug treated group. Concomitant treatment of mice with $5x10^4$ units of rhIFN-α and 454A12 MAB-rRTA (10μg) resulted in 35% long term survivors as compared with 19% survivors in mice that received the same dose of IT alone. Even though there appeared to be a trend towards increasing the MST in the group of mice treated with the IT plus rhIFN-α when compared to IT alone, this trend was not statistically significant ($p=.064$). When these studies were terminated at 150 days, long term survivors were observed to be free of malignant ascites. However, solid

peritoneal tumor growth was evident in the majority of mice at necropsy indicating tumor cell kill was incomplete. This existence of tumor implants may be due to the failure of the IT to penetrate more than several cell diameters due to poor vascularity of the tumor. We are currently investigating the use of specific ITs constructed from $F(ab')_2$ fragments in combination with rhIFN-α. Such a low molecular weight conjugate may distribute more efficiently within the tumor mass.

PUBLICATIONS

Pearson JW, Hedrick E, Folger 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 α-interferon. Cancer Res 1990;50:6379-88.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09322-03 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
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COOPERATING UNITS (if any): Biological Carcinogenesis and Development Program, PRI/DynCorp, NCI-FCRDC (T. J. Sayers); Stanford University (E. C. Butcher); Harvard Medical School (M. P. Bevilacqua, A.K. Bhan); American Red Cross (T. Maciag)

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.)

Following the treatment of mice with biological response modifiers, we have observed a recruitment of cells with natural killer (NK) activity to the liver and spleen, presumably in response to the local release of cytokines or other mediators. To test whether specific cytokines could stimulate migration of NK cells, we used a modified Boyden chamber assay to study their chemotaxis in vitro. Recombinant human (rh) interleukin (IL)-2, rhIL-6 and rh tumor necrosis factor α (TNFα) induced directed migration of purified rat NK cells in a dose and time-dependent fashion. In contrast, rhIL-1α, rhIL-8, r rat interferonγ (IFN-γ) and rhTNFβ were inactive in this assay, suggesting that regulation of NK recruitment involves responses to specific inflammatory cytokines. Splenic NK cells and the murine vascular endothelial cell line eEND-2 were used to study the effects of cytokines on NK cell adhesion and penetration through capillary endothelial barriers. Treatment of eEND-2 cells with 10-1000 U/ml of rmTNFα resulted in a 4-7 fold increase in NK cell adhesion. rhIL-1 treatment of eEND-2 cells also increased NK cell adhesion 2-4 fold over background levels. In contrast, treatment of eEND-2 with either rhIL-6, rhIL-7 or rmIFN-γ had no effect on NK cell adhesion at any of the doses tested. Pretreatment of the NK cells alone with the cytokines produced only a modest increase in their adhesiveness, suggesting that modulation of surface adhesion molecules on eEND-2 cells by rmTNFα is responsible for the enhanced effects. Future studies will focus on the nature of these molecules and their counter-ligands present on NK cells. Taken together, these results suggest that local release of cytokines in tissue sites following BRM treatment may regulate the recruitment and activation of leukocyte subsets in vivo. To test this hypothesis, isolated rat Kupffer cells were treated with various BRM or cytokines and examined for cytolytic activity against rat hepatoma cells. Only treatment with rhIL-2 was capable of activating rat Kupffer cell cytotoxicity directly, suggesting that different signals are required to stimulate leukocyte recruitment and activation.

PROJECT DESCRIPTION

PERSONNEL

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Patricia S. Latham	Biotechnology Fellow	IS, LMI, 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 induction of directed leukocyte migration or chemotaxis, by defined cytokines using an in vitro Boyden chamber assay. 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 study the ability of BRM-induced cytokines to cause chemotaxis of LGL and other leukocyte subsets.

2. To determine the signals which cause LGL and other leukocyte subsets to accumulate in vivo after cytokine treatment.

3. To study the interaction of leukocytes with endothelial cells and basement membrane following treatment with BRMs.

4. To characterize and identify soluble factors released from BRM-treated liver cells which may mediate the activation/localization of cytolytic effector cells observed in vivo.

RESULTS

Inflammation is characterized by an infiltration of mononuclear cells, accompanied by the local production of inflammatory cytokines, such as IL-1, IL-6, IL-8 and TNFα. We have previously shown that injection of specific cytokines, or combinations of cytokines, can mediate the recruitment of different populations of leukocytes into tissue sites. Similarly, treatment of mice or rats with various BRM results in increases in both the number and cytolytic activity of large granular lymphocytes (LGL) with NK activity in the liver and spleen. Thus, cytokines or other mediators released in these organs following BRM treatment may be responsible for the recruitment and/or activation of LGL observed. We have been studying the role of these factors in mediating the migration of LGL in an in vitro Boyden chamber assay.

Recombinant human (rh) cytokines, including rhIL-2, rhIL-6 and rhTNFα were found to induce directed migration, or chemotaxis of rat LGL in a dose-dependent fashion. rhIL-2 was the most active cytokine tested in stimulating LGL movement, with a 2-3 fold increase in migration observed at concentrations as low as 10-100 U/ml, and reaching a maximal effect at 10,000 U/ml. Neither rhIL-1 nor rhIL-8 were capable of stimulating LGL movement at any concentration tested. In contrast, rhTNFα induced migration of LGL in a bell-shaped dose-response, with maximal effects (2-3 fold) observed when 100-300 U/ml were placed in the lower wells. Recombinant murine TNFα also stimulated LGL movement in a similar manner. Similar concentrations of lymphotoxin inhibited LGL migration when placed in the lower wells. These results suggest that TNFα may be an active mediator in inducing LGL locomotion into inflammatory sites.

Migratory leukocytes must be able to bind to and penetrate capillary endothelial cells and their underlying basement membrane before extravasation into tissue sites can occur. Recently, 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.

We have obtained a murine endothelial cell line, eEND2, from Dr. Eugene C. Butcher (Stanford University) to develop our model of murine NK-endothelial cell interaction. This line was derived from a polyoma-virus induced hemangioma in male ICR mice, and displays many similar characteristics to cultured human umbilical venule cells. We have demonstrated that quiescent eEND2 cells form confluent monolayers, tight junctions between cells, and adhere to plastic microtiter dishes in the absence of any matrix material, suggesting that they are capable of forming intact models of the endothelial component of the microvasculature in vitro.

With the ability to generate confluent monolayers of mouse endothelial cells, we now have begun to study interactions between these cells and cytotoxic effector cells. Our initial studies examined the interaction of murine splenic NK cells with eEND2 cells. Because of the difficulties involved in isolating large quantities of murine NK cells in sufficient purity, we have developed an assay to quantitate NK cell numbers by measuring their granzyme A serine esterase activity. In collaboration with Dr. Thomas Sayers (BCDP, PRI/DynCorp), we initially correlated the amounts of granzyme A esterase activity with the numbers of NK1.1 positive splenocytes obtained from C57BL/6 nu mice. Increasing numbers of NK1.1 positive cells were solubilized in 0.5% NP-40, and their granzyme A activity measured following the reduction of the substrate Nα-carbobenzoxy-L-lysine thiobenzyl ester (BLT). A positive linear

correlation between the amounts of extracted BLT-esterase activity and the number of NK1.1 positive splenocytes, as measured by flow cytometry was obtained ($r = 0.993$). Extracted eEND2 cells, enriched B cells, thymocytes or resident peritoneal macrophages had no demonstrable BLT-esterase activity present, allowing us to use this enzyme activity as an index of NK cell adherence.

Initially, we examined the effects of specific cytokine treatment of eEND2 cells on murine NK adherence. When eEND2 cells were stimulated with 100 U/ml of either rhIL-1α, rmuTNFα or crude supernatants from concanavalin A treated splenocytes, adhesion of NK cells was increased 2-4 fold over background levels. TNFα treatment of eEND2 cells caused the most pronounced increase in NK cell adhesion, with between 30-70% of the input cells adhering after 60 min. These effects were dose-related, reaching a maximal enhancement at concentrations of TNFα between 10 and 1000 U/ml. Increases in NK adhesion to eEND2 cells were also dependent on the time of exposure to cytokines, with a 2 hr pre-treatment resulting in maximal effects, reaching a plateau at 4-6 hr and declining with overnight pre-treatment. In contrast, stimulation of endothelial cells with regimens that enhance T cell binding, such as rmuIFN-γ or rhIL-6, failed to enhance NK cell adherence, suggesting that NK cells and T cells recognize and adhere to stimulated endothelium via different surface receptors. Treatment of NK cells alone with the cytokines produced only a modest increase in adhesiveness, suggesting that modulation of surface adhesion molecules on endothelial cells by TNFα is responsible for the enhanced effects. Studies will be conducted to examine these interactions using specific monoclonal antibodies directed against adhesion molecules found on both endothelial and effector cells. Using commercially available monoclonal antibodies against murine NK cells, or rabbit antisera generated against endothelial cells (Dr. Michael P. Bevilacqua, Harvard Medical School), we will study which subclasses of adhesion molecules are important in regulating NK cell interactions with stimulated endothelial cells. We will also attempt to generate polyclonal antisera against murine endothelial cell adhesion molecules specific for NK cell interactions. Future studies are also planned to investigate the penetration of endothelial cell monolayers and the underlying basement membrane in vitro. Using commercially available transwell chambers, 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 transwell 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 used to help overcome the current limitations of cytokine and/or adoptive immunotherapy in mice and humans.

Our initial studies demonstrated activation of NK cells in the liver and the spleen following treatment of mice with BRM, however only the liver showed appreciable increases in NK cell number. Additionally, the endothelium in the liver is sinusoidal in nature, and the cells themselves are fenestrated. These results suggest that differences in regional endothelial cell physiology

between various organs may influence the accumulation of specific leukocyte subsets in response to local stimuli. To examine this possibility, we have attempted to isolate and grow large numbers of murine and rat endothelial cells from specific organ sites, such as the liver, spleen and peritoneal wall. Using collagen sponges saturated with the angiogenic fibroblast growth factor α (FGFα), we have generated vascular beds in vivo that originate from various organs, but are devoid of parenchymal cells. Sponges containing the vascular endothelium may then be surgically removed and digested with collagenase to yield primary isolates of endothelial cells. Primary rat liver endothelial cells were also recovered by centrifugal elutriation of non-parenchymal cells isolated from perfused liver digests. Both techniques have yielded reasonable numbers of endothelial cells with purities of approximately 50%. Endothelial cells were further enriched using flow cytometric techniques and cell sorting to a final purity of 95%, however, this step reduced the viability of these cells to such a low level as to be impractical. Viable rat liver endothelial cells were obtained from approximately half of the elutriations, however, these cells were extremely fragile and did not survive trypsinization required for passage. Cultures of primary isolates of much lower purities were successfully grown in the presence of FGFα, however, these were eventually overgrown by contaminating fibroblasts and other non-endothelial cells.

To minimize the contamination by other cell types, we have tried several methods to either selectively promote endothelial attachment or to inhibit fibroblast proliferation. Fibronectin-coating of tissue culture flasks yielded the most favorable conditions for endothelial cell growth, however, fibroblastic proliferation was also enhanced. Similar results were obtained when collagen type IV was used to treat tissue culture flasks, and we found that endothelial cells could not be induced to proliferate on laminin-treated flasks. To overcome the problem of fibroblastic overgrowth, we have treated cultures with the hydroxyproline analog cis-(allo)-hydroxyproline. This analog substitutes for hydroxyproline during collagen biosynthesis, resulting in an inhibition of fibroblast proliferation due to buildup of collagen precursors. We found that addition of this compound to primary cultures of mouse endothelial cells could retard the growth of fibroblasts, but these cells eventually adapted and overgrew the endothelial cell monolayers. Future cultures of organ-specific endothelial cells will be plated into artificial basement membrane substrates (Matrigell), which do not support fibroblastic growth. In collaboration with Dr. Thomas Maciag (American Red Cross), we will also utilize NIH/3T3 cells that have been transfected with the gene for FGFα fused to a signal sequence which allows secretion of the protein. These cells, when implanted on a suitable support (Gortex) invoke a massive angiogenic response resulting in de novo large vessel formation. Vessels generated by this approach may provide such large numbers of endothelial cells from specific organ sites without the need for further purification.

Since we have previously demonstrated that injection of mice with cytokines or BRM can also lead to activation of NK and other effector cells in situ, we extended our studies to include the effects of these agents on the activation of effector cells for tumor cytolysis. Because treatment of tumor bearing mice with BRM can result in a significant decrease in metastases to the liver,

we have focused our studies on the activation of liver macrophages (Kupffer cells) by BRM and rh cytokines. In collaboration with Dr. Patricia Latham (LMI), we have examined the effects of in vitro treatment of rat Kupffer cells with endotoxin (LPS), or the cytokines r rat IFN-γ and rhIL-2. Kupffer cells treated with IL-2 showed dose-dependent increases in cytolytic activity towards a rat N1S1 hepatoma, as well as towards P815 and Yac-1 murine tumor targets. In contrast, treatment with combinations of IFN-γ and LPS did not enhance Kupffer cell cytotoxicity as well as IL-2 treatment. Using flow cytometry, we have demonstrated that a small (8-12%) subpopulation of NK cells also resides in the liver. Since these cells may also be activated by IL-2, we used the anti-rat LGL antibodies OX-8 or 3.2.3 plus complement to deplete liver NK cells, however, this regimen proved toxic to the macrophages as well. Future studies using centrifugal elutriation to enrich for Kupffer cells are planned to avoid these problems. We also plan to utilize the newly developed monoclonal antibody KCA-3 (Dr. Arna K. Bhan, Harvard Medical School) to positively select for Kupffer cells using flow cytometry and cell sorting techniques. These procedures will allow us to definitively determine which subpopulation is responsible for the enhanced cytotoxicity following IL-2 exposure.

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;3:219-32.

Latham PS, Pilaro AM, Echeagaray PL, Varesio, L. Interleukin-2 (IL-2) activates rat Kupffer cells (KC) to express cytotoxicity in vitro. In: Kirn AC, Knook DL, Schleister EC, Wisse E, eds. Proceedings of the meeting Immunology of the Liver. Washington, D.C.: American Association for the Study of Liver Disease, 1991;in press.

Pilaro AM, Sayers TJ, McCormick KL, Reynolds CW, Wiltrout RH. An improved in vitro assay to quantitate chemotaxis of rat peripheral blood large granular lymphocytes (LGL). J Immunol Meth 1990;135:213-24.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09348-02 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.): Chemoprotective Effects of Recombinant Cytokines

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

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Others:	G. Damia	Visiting Fellow	LEI, NCI
	M. MacPhee	Visiting Fellow	LEI, NCI
	N. Usui	Visiting Fellow	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, PRI/DynCorp, 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: 4.0	PROFESSIONAL: 3.0	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.)

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. Subsequently, further studies have demonstrated that the administration of multiple doses of $TGF\beta_1$ via the ip or iv routes has profound effects on the proliferation of progenitor cells. IL-1 also has myeloproliferative and chemoprotective effects. The daily (5-7 days) administration of rIL-1α (≥ 10,000 U/day) protects 70-100% of mice from acutely toxic doses of several chemotherapeutic drugs. Current studies are focused on 1) the cellular and molecular mechanisms for the hematological and chemoprotective effects of rTGFβ1 and rIL-1α and 2) further investigation into improved therapeutic efficacy of intensified chemotherapy.

PROJECT DESCRIPTION

PERSONNEL

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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 recombinant tumor growth factor β_1 (rTGFβ_1) and recombinant interleukin-1α (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. rTGFβ_1 has been shown to inhibit bone marrow (BM) colony formation following in vitro treatment as well as after in vivo administration to normal mice. These data suggest that TGFβ might either protect, or further depress, stem cell levels in mice exposed to a cell cycle-active drug such as 5-fluorouracil (5FU). Recombinant rTGFβ_1 was administered either as a single dose (5 μg/mouse) intra-arterially (ia) or as repeated doses intravenously (iv) or intraperitoneally (ip) to mice during the hyperproliferative state of the bone marrow that occurs 7-9 days after the iv administration of 150 mg/kg 5FU. In both instances, the formation of multilineage (CFU-GEMM) colonies was more inhibited (51%) than the more differentiated (CFU-c) colonies (31%). Surprisingly, pretreatment with a single ia dose of rTGFβ_1 24 hours before the administration of 5FU also partially inhibited the subsequent increase in both CFU-c (13%) and CFU-GEMM (27%) that occurred by about 9 days. However, when multiple doses of rTGFβ_1 were administered systemically immediately prior to the injection of 5FU, the rebound in CFU-c and CFU-GEMM colonies was markedly inhibited by 40-77% and 44-77%, respectively. This effect was maximal when rTGFβ_1 was administered at daily doses of $\geq$ 5 μg/mouse for at least 3 days. This inhibition of the recovery of the bone marrow from 5FU treatment induced by rTGFβ_1 was a delayed

transient response since by day 16 the progenitor cell numbers and bone marrow cellularity were identical to the 5FU-treated marrow control.

IL-1 is a multifunctional cytokine that also plays 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 several chemotherapeutic drugs. Specifically, the pretreatment of mice with recombinant human interleukin 1α (rhIL-1α) protects mice from the lethal effects of several myelotoxic chemotherapeutic drugs, including 5-fluorouracil (5FU), cyclophosphamide (Cy), carboplatin (JM8), and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). Pretreatment with rhIL-1α was not effective against the acute lethal toxicity generated by adriamycin (ADR) and cisplatin (DDP). The chemoprotective effects appear to be at least partially due to myelo-protection/restoration since the recovery of colony forming units (CFU) and total while blood cell counts was accelerated in the rhIL-1α-pretreated mice. However, the chemoprotective effects of rhIL-1α are apparently not limited to myeloprotection since pretreatment with rhIL-1α protects mice against the lethal toxicity of both 5FU and Cy, yet bone marrow transplants rescue mice treated with 5FU but not those treated with Cy. The chemoprotective effects of rhIL-1α may be at least partially indirect since the efficacy of chemoprotection by rhIL-1α is reduced in athymic mice, and interleukin 6, but not TNFα, can substitute for rhIL-1α for chemoprotection for 5FU. 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. 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 development of overt toxicity. 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 cyclophosphamide. We have utilized this technique, supported by

immunohistochemical studies of the lungs of effected animals, to determine that T cells appear to be required for the development of Cy-induced fibrosis, since the amount of fibrosis that occurs in athymic mice is less than that which develops in euthymic mice. Further studies are in progress to identify the cells involved in the development of fibrosis, and 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.

PUBLICATIONS

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SUMMARY REPORT

LABORATORY OF BIOCHEMICAL PHYSIOLOGY

October 1, 1990 through September 30, 1991

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 novel laboratory 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, developmental biology and microinjection techniques to the studies of interleukin-6 (IL-6) and its receptors, ras and other guanyl nucleotide-binding proteins (G-proteins), phospholipase C (PLC), protein kinase C (PKC), and transcription and translation factors. Recently, the LBP has established an early embryo system to study differential participation of ventral and dorsolateral mesoderms in the hemopoiesis of Xenopus. The system will be used to study the roles of various BRMs in the differentiation of hemopoietic cell lineage in Xenopus embryos.

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. In the current studies, our research effort has been focused on the further characterization of the GDP/GTP exchange factor of 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 stimulation of 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 the mechanism for interconvertion of 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. We refer to this novel factor as ras guanine nucleotide exchange factor (rGEF). In studies of the response of ras activity to growth factor, we have shown the accumulation of active GTP-p21 complex in serum-stimulated NIH 3T3 cells. Modulation of rGEF and ras GTPase activating protein (GAP) activity would be expected to play an important role in ras-mediated transformation. Molecular cloning and functional studies of rGEF are in progress.

MITOGENIC PROPERTIES OF EUKARYOTIC INITIATION FACTOR-4E (eIF-4E)

Recently, we have shown the induction of DNA synthesis and morphological transformation after microinjection of eIF-4E into NIH 3T3 cells. eIF-4E could conceivably represent a novel class of oncogenic proteins. The mitogenic activity of eIF-4E, as measured by DNA synthesis, was stimulated by co-injection with PKC and inhibited by co-injection with neutralizing anti-ras antibody. These results demonstrate the functional relationship among PKC, ras, and eIF-4E proteins. In addition, we have obtained preliminary results on the suppression of eIF-4E mitogenic activity by protein kinase A. Our studies demonstrate a biological link between mitogenic activity and protein phosphorylation at a translational level.

THE SIGNAL TRANSDUCTION PATHWAYS IN XENOPUS LAEVIS

We have been using Xenopus oocytes as a model system to study the biochemical mechanisms of signal transduction pathways. cDNAs for many components involved in signal transduction have been cloned in our laboratory. The cloning work has set the stage for the functional studies of those components. Oocyte microinjection techniques have been employed for the functional studies. Recently, our studies have been extended beyond oocyte maturation leading to a better understanding in amphibian embryogenesis. We have established an early Xenopus embryo tissue culture system to study differential participation of ventral and dorsolateral mesoderms in the hemopoiesis of Xenopus. In studies of the effects of exogenous factors on the differentiation of hemopoietic cells, we have shown that the addition of TGF-β1 (not TGB-β2) to the tissue culture stimulates the hemopoiesis of ventral mesodermal tissue from the early gastrula (stage 10).

INTERLEUKIN-6 (IL-6) AND ITS RECEPTORS

By affinity cross-linking, we have shown earlier that myeloma cells express a high affinity IL-6 receptor (p110), in addition to the previously cloned p80 IL-6 receptor. The current studies have been directed towards establishing with some degree of certainly whether p110 and p80 are proteins encoded by different genes. Recently we have obtained p80 antibodies and a full-length cDNA clone from Dr. T. Kishimoto. The availability of these reagents will facilitate further characterization of the p110 IL-6 receptor.

In addition to the biochemical studies of IL-6 receptor, we have been investigating IL-6-mediated biological responses. We have demonstrated that IL-6 stimulates the secretion of at least five proteins into culture medium of a responsive human melanoma cell line (A375-C6). Two secreted proteins have been identified as extracellular matrix proteins, fibronectin and tenascin. The relationship between the IL-6 antiproliferative effect in A375-C6 cells and the IL-6-induced secretion of extracellular matrix proteins will be investigated.

AIDS-RELATED RESEARCH

We have been working on the transcriptional regulation of human immunodeficiency virus (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 the HIV-infected monocytoid leukemic cell line, THP-1. DNA-binding gel retardation assays and Southwestern blot assays have been used to study the molecular mechanisms of HIV gene regulation in monocytes/macrophages. 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. In addition to THP-cells, we have also been working with primary monocytes/macrophages. 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, several inhibitors of HIV have been isolated and purified to homogeneity from the Chinese medicinal plants, i.e. MAP30, TAP29, GAP30, DAP30, and DAP32. They exhibit 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 they are potential therapeutic agents in the treatment of HIV-1 infection. The N-terminal amino acid sequences of these proteins have been determined. Molecular cloning of the genes encoding for these proteins is in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09299-05 LBP

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.)*

Characterization of HIV Infection in Peripheral Blood Mononuclear Cells

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	W. P. Tsai	Microbiologist	LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, 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:
4.0	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.)*

We have been performing the following studies: 1. cytokine production by monocyte/macrophage persistently infected with HIV; 2. quantitative infectious cell center (ICC) assays for detection of peripheral blood mononuclear cells (PBMC) infected with HIV; and 3. characterization of monocyte/macrophage-tropic viruses. The results show that although HIV infection itself induced significant levels of mRNAs for IL-1β, TNF-α, IL-6, and IL-8, the levels of lipopolysaccharide (LPS)-induced mRNAs for IL-1α, IL-1β, TNF-α, IL-6, and IL-8 in HIV-infected human cultured macrophages were much lower than those in uninfected LPS-stimulated human macrophages. Consistent with the IL-8 mRNA expression data, the HIV-infected macrophages produced a much lower amount of IL-8 protein, as measured by a radioimmunoassay, than uninfected LPS-stimulated cells over an 18-day culture period. These results suggest that HIV-infection generally suppresses the inducible cytokine production in human macrophages. In our studies using ICC assay, PBMC that are infected with a rapid/high cytopathic virus (HIV-1RF) form discrete syncytia by co-culturing with a CD4-positive cell line in microtiter plates. Similar results were observed with monocytes/macrophages. Further studies will be performed with slow/low, as well as other rapid-high isolates in this system. We have also determined the growth kinetics of a monocyte-tropic virus infecting monocytes/macrophages by the detection of viral protein components. Preliminary results showed that gp120 was dissociated from virus particles.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Wen-po Tsai	Microbiologist	LBP, NCI

INTRODUCTION

HIV has been isolated from two primary human immune cells, $CD4^+$ T cells and monocytes/macrophages. Viruses isolated from various tissues appear to be heterologous in cell tropism in vitro. In order to understand the pathogenesis of the virus with discrete cell tropism and consequently to develop prevention/therapy of AIDS, we have been studying the following three aspects of the virus-host cell interaction using human primary peripheral blood lymphocyte and monocyte/macrophage cultures. 1. Cytokine production by monocyte/macrophages persistently infected with HIV and 2. quantitative infectious cell center assays for the detection of PBMC infected with HIV. These assays would be particularly useful for the detection of slow/low cytopathic viruses. These viruses, which are most often isolated from asymptomatic stages of AIDS and many of which lack syncytial-forming capacity in cell lines, have been only poorly characterized due to lack of similar infectivity assays for rapid/high cytopathic virus, and consequently their roles in the disease progression are largely undetermined. 3. Characterization of the monocyte/macrophage-tropic viruses; i.e. growth kinetics in macrophages, the production of major viral components including a core protein (p24), reverse transcriptase (RT), surface glycoprotein (gp120), infectivity of $CD4^+$ lymphocytes and monocytes/macrophages.

MAJOR FINDINGS

1. The capability of the production of several immunomodulating cytokines including interleukin-1α (IL-1α), interleukin-1β (IL-1β), TNF-α, IL-6, IL-8, and macrophage chemo-attractant and activating factor (MCAF) by human monocytes/macrophages infected with HIV-1 RF isolate was examined in vitro. Although HIV infection itself induced significant levels of mRNAs for IL-1β, TNF-α, IL-6, and IL-8, the levels of lipopolysaccharide (LPS)-induced mRNAs for IL-1α, IL-1β, TNF-α, IL-6, IL-8, and MCAF in HIV-infected human cultured macrophages were much lower than those in uninfected LPS-stimulated human macrophages. In addition, the HIV-infected macrophages produced much lower amounts of IL-8 protein as measured by radioimmunoassay than uninfected LPS-stimulated cells over an 18-day culture period. These results suggest that HIV-infection generally suppresses the inducible cytokine production in human macrophages.

2. In our studies, peripheral blood lymphocytes that were infected in vitro with a well-characterized rapid/high cytopathic virus (HIV-1 RF), form discrete syncytia by co-culturing with a clone $CD4^+$ cell line in microtiter plates. Similar results were observed with monocytes/macrophages after one hour of incubation with HIV-1 RF.

3. Our preliminary studies show that human monocyte/macrophages infected with a monocyte-tropic HIV-1 (ADA) produce a virus as measured by the expression of p24, RT, and gp120 in culture media. p24 and RT appeared approximately 2 weeks after infection and reached a peak level after the third week. The profile of the gp120 synthesis appeared to be different. This protein was detected after the first week and reached the peak level during the second week; afterwards the level stabilized. It is most interesting to note that gp120 was not detected in the virus pelleted by ultracentrifugation, indicating that this protein was not associated with the virus particle.

OBJECTIVES AND FUTURE PLANS

Our objectives and future plans are as follows:

1. We will further study slow/low, as well as other rapid/high isolates utilizing the quantitative infectious-cell center assay with PBMC. The system would facilitate HIV studies in many aspects, particularly for characterization of slow/low viruses regarding titration of infectivity, the origins, neutralizing epitopes, sensitivity to antiviral agents, their roles in disease progression, and finally for designing effective vaccines and antiviral agents.

2. We have preliminarily determined the growth kinetics of the monocyte-tropic virus infecting monocytes/macrophages by the detection of viral protein components. We will further study the infectivity of the virus in primary lymphocytes and macrophages in relationship to the expression of viral proteins, particularly gp120, and will characterize the neutralizing epitopes of these viruses.

3. We will elucidate the roles of cytokines in the pathogenesis, as well as prevention/therapy of HIV-infection.

PUBLICATIONS

Lee-Huang S, Huang PL, Nara PL, Chen HC, Kung HF, Huang P, Huang HI, Huang PL. MAP 30: A new inhibitor of HIV-1 infection and replication. FEBS Letter 1990;272:
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Lee-Huang S, Huang PL, Kung HF, Li BQ, Huang PL, Huang P, Huang HI, Chen HC. A new anti-HIV protein from trichosanthes, nontoxic to intact cells. Proc Natl Acad Sci USA 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09300-05 LBP

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.)*

Intracellular Activities of Cytokines

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	S. K. Durum	Senior Staff Fellow	LMI, NCI
	L. Bristol	IRTA Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, NCI-FCRDC (M. Smith, Y. Liu)

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NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	0.0	4.0

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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.)*

Polypeptide hormones bind to cell-surface receptors and generate second messenger molecules that regulate the cellular metabolism. In addition, the receptor-ligand complex is internalized by receptor-mediated endocytosis and localized within endosomes and lysomes in the cytoplasm of the cell. We postulated that the internalized ligand may have an intracellular role and tested this idea by directly injecting cytokines into the cytoplasmic compartment. Microinjection of interferon-γ (IFNγ) was shown to induce Ia expression across the species barrier and injection of TNFα induced rapid cytotoxicity and DNA fragmentation. Recently, injection was used to identify a role for PKC in the signal transduction leading to surface Ia expression on macrophages, a biological response seen normally from IFNγ treatment.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Scott Durum	Senior Staff Fellow	LMI, NCI
Lynn Bristol	IRTA Fellow	LPPS, NIAA

INTRODUCTION

The mechanism of intracellular cytokine-induced signaling is important in order to gain a general understanding of how polypeptide hormones signal metabolic changes in cells. Previously, we have demonstrated that human IFNγ after microinjection into murine macrophages induced class II antigens on the cell surface irrespective of the species barrier that restricts the receptor-mediated activity of IFNγ. This observation suggested that IFNγ has an intracellular role in Ia expression that is not species-specific. In order to understand the intracellular aspects of cytokine-induced signaling, we have used microinjection to investigate two of the well characterized signaling pathways, protein kinase A (PKA) and protein kinase C (PKC). Purified components of PKA and PKC signaling pathways have been injected into macrophages to evaluate the intracellular activity of these enzymes on the induction of class II antigens (Ia) at the cell surface. We have demonstrated that phospholipase C-γ (PLC-γ), ras protein, and PKC induce Ia antigen presentation on the cell surface 48 hours after injection, while PKA and phospholipase A_2 (PLA_2) do not have this activity. These results suggest that the PKC pathway controls the expression of genes in the major histocompatibility complex and may play many other roles in the activation of macrophages.

We have continued the investigation regarding the intracellular roles of TNFα. TNFα was shown to induce apoptosis after injection into the confluent monolayers of fibroblast cells. Recently, we have demonstrated a role for PKC and ornithine decarboxylase (ODC) in this TNFα-mediated cytotoxicity. We have also demonstrated that the activation of a Ca^{++}-dependent endonuclease may be responsible for the characteristic fragmentation of genomic DNA seen with the TNFα killing of cells.

OBJECTIVES

The goals of this project are: 1. to study the biological activities of cytokines after injection into specific target cells and to compare the intracellular biological response to the usual receptor-mediated response observed after treatment with cytokines; and 2. to elucidate various signaling pathways via injection and biochemical analysis.

METHODS

The following techniques were used: 1. microinjection; 2. tissue culture; 3. protein purification; 4. immunological staining; 5. enzyme assays; and 6. DNA fragmentation.

MAJOR FINDINGS

Unstimulated peritoneal macrophages grown in culture can be virtually 100% activated for Ia expression by treatment with IFNγ, and phorbol esters (PMA) display this same activity with an efficiency of 50-60%. When metabolic inhibitors of PKC (staurosporine, H7, and calphostin C) are added to the culture media before IFNγ or PMA, the percentage of cells expressing Ia is reduced to 40% and 9% (background levels), respectively. This result suggests that at least part of the IFNγ signal and all of the PMA signals are mediated through PKC-related metabolic pathways. To further substantiate the role of PKC in Ia gene expression, purified PKC was microinjected into the cytoplasm of macrophages. As a result of this injection, 45% of the injected cells expressed Ia after a 48-hour incubation. In addition, other intermediates (PLCγ and ras protein) that may lead to PKC activation also induced Ia expression. In contrast intermediates of the PKA metabolic pathway (PLA_2 and PKA itself) did not induce Ia. Thus, the PKC pathway can control gene expression of the major histocompatibility complex and may also play other roles in macrophage activation.

TNFα has been shown to be a growth factor for fibroblast cells and is thought to activate a suicide pathway in many cell types that results in DNA fragmentation and cell death. We have demonstrated that PKC reduces the efficiency of TNFα-mediated killing of fibroblast cells and have characterized an increased endonuclease activity which correlates with TNFα killing. These experiments link PKC and endonuclease activity to TNFα-mediated cytotoxicity.

FUTURE PLANS

Our future plans include: 1. to continue studying the mechanism of signal transduction in macrophages by injection of purified components; 2. to compare classical receptor binding signaling to the intracellular activity of cytokines; and 3. to purify and clone the TNFα activated endonuclease.

PUBLICATIONS

Bristol LA, Smith MR, Bhat NK, Durum SK. IL-1 induces ornithine decarboxylase in normal T lymphocytes. J Immunol 1991;5:1509-15.

Chuang LP, Zhao FK, Kung HF, Israel M, Chuang RY. N-trifluoroacetyladriamycin-14-valerate (AD32) blocks protein kinase C activation by tumor promoters. Cancer Research 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09301-05 LBP

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.)*

Transforming Properties of Eukaryotic Initiation Factor-4E

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

PI: H. F. Kung Chief LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, NCI-FCRDC (M. Smith, Y. Liu)

LAB/BRANCH

Laboratory of Biochemical Physiology

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INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	0.0	4.0

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.)*

Several proto-oncogenes have been designated as intermediates in signal transducing pathways and have been categorized by structural/functional criteria and biological activity into four groups: 1. growth factors and their receptors; 2. signal transducing G-proteins; 3. cytoplasmic serine/threonine protein kinases; and 4. nuclear factors involved in transcriptional regulation. Recently, we have identified a new class of potential oncogenes. The translational initiation factor 4E (eIF-4E) has been shown to induce tumors in nude mice and induce DNA synthesis and morphological transformation after microinjection into NIH 3T3 cells. The mitogenic activity of eIF-4E as measured by DNA synthesis was enhanced 5-fold by co-injection with PKC and inhibited by co-injection with neutralizing anti-ras monoclonal antibodies. These results demonstrate a direct biological link between PKC and eIF-4E activity and also demonstrate a requirement for G-protein signal transduction in the activation of eIF-4E bioactivity.

PROJECTION DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI

INTRODUCTION

Microinjection has been used to characterize the oncogenic potential of eIF-4E. eIF-4E is a 24 kDa polypeptide that binds to the 5' mRNA cap site and is a component of the trimeric initiation factor 4F. eIF-4F contains two other peptides, eIF-4A (a 46 kDa protein that binds and hydrolyses ATP and displays RNA helicase activity) and 220 kD protein, with an unknown function whose integrity is required for eIF-4F activity. The biological activity of eIF-4E has been shown to correlate to its phosphorylation, suggesting a link between translational efficiency and the protein kinase cascade.

OBJECTIVES

The objectives of this project are to characterize the transforming properties of eIF-4E and to establish a link among G-proteins, PKC signal transduction, and eIF-4E bioactivity.

MAJOR FINDINGS

Injection of recombinant eIF-4E or rabbit reticulocyte purified eIF-4F into quiescent NIH 3T3 cells induced a dosage-dependent induction of DNA synthesis (12-18 fold) above that observed from injection of mutant eIF-4E(Ala-53) or other purified initiation and elongation factors. In addition, eIF-4E and eIF-4F induced a morphological alteration that was visible 6-8 hours after injection and was apparent for approximately 30 hours. This morphological transformation was not observed after injection of other initiation and elongation factors. The profile of the eIF-4E dose response curve and the lack of biological activity from the eIF-4E(Ala-53) mutant suggested that some intracellular activity may be limiting in the injected quiescent NIH 3T3 cells and that this activity may relate to the phosphorylation of eIF-4E. To test this hypothesis eIF-4E was co-injected with protein kinases. Co-injection of PKC with minimal concentrations of eIF-4E resulted in a 5-fold enhancement of DNA synthesis activity. The enhancement effect was not seen with co-injection of PKA or the raf protein. Addition of PMA to the culture media of eIF-4E injected cells also resulted in increased DNA synthesis, supporting the role of PKC in the observed effect. eIF-4E activity is thought to be the overall rate-limiting step in translation, in that eIF-4E is found in cells at approximately an order of magnitude less than other initiation and elongation factors. The mechanism by which eIF-4E acts to transform cultured cells after injection is not known but may relate to an increased ability to form the eIF-4F complex and enhance translation rates. There are two possible explanations for this enhancement. First, the cells may be demonstrating an overall increase in mRNA translation,

in which case eIF-4E would be acting as a nonspecific promoter of mitogenesis. Second, there may be a specific subclass of mRNA that exhibits greater sensitivity to regulation by eIF-4E. This class of mRNA may have extensive 5' noncoding secondary structure. These observations support a mechanism whereby eIF-4E regulates the translation of specific mRNAs that code for key components in the metabolic pathways associated with cell growth, differentiation, or neoplasia. In preliminary experiments, we have demonstrated that the mitogenic activity of eIF-4E requires ras G-protein signal transduction. Co-injection of eIF-4E and the neutralizing anti-ras monoclonal antibody, Y13-259, results in blockage of DNA synthesis induction, while the control antibody, Y13-238, has no effect.

FUTURE PLANS

Our future plans include: 1. to confirm the ras antibody experiments with microinjection of dominant inhibitory ras mutant proteins (substitution of asn for ser at position 17 of ras) and 2. to complete our preliminary studies showing the down-regulation of eIF-4E mitogenic activity by PKA.

PUBLICATIONS

Smith MR, Jaramillo M, Tuazon PT, Traugh JA, Liu YL, Sonenberg N, Kung HF. Modulation of the mitogenic activity of eukaryotic translational initiation factor-4E by protein kinase C. The New Biologist 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09302-05 LBP

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.)*

Studies on the Biological Functions of Human Ras Proteins

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	M. Maeno	Visiting Fellow	LBP, NCI
	J. Gopas	Special Volunteer	LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, NCI-FCRDC (M. West, B. Li, T. Kamata)

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	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.)*

Mammalian ras proteins bind guanine nucleotides and have been implicated in the malignant cell transformation, as well as in the control of cellular proliferation and differentiation. We have recently identified and purified ras guanine nucleotide exchange factor (rGEF) which is capable of stimulating the GDP/GTP exchange reaction of ras proteins. Studies were performed to establish the physiological role of rGEF in ras-mediated signal transduction.

In order to conduct the molecular biological studies of rGEF functions, the molecular cloning of cDNA encoding rGEF is in progress. For the immunological screening of the cDNA library, we are producing the monoclonal antibodies against rGEF. The domain of ras proteins involved in the interaction with rGEF was also investigated by the mutational analysis. Our data indicated that 58-68 amino acid residues of ras protein may be responsible for the interaction.

In another approach, we analyzed the response of ras activity to the growth signal. The active ras-GTP complex was accumulated in serum-stimulated NIH 3T3 cells. It is possible that rGEF and/or GAP play important regulatory roles in ras-mediated cell proliferation/differentiation. Also, we have developed an in vitro reconstitution system. These studies will provide a further insight into the regulatory mechanism of ras activity in transmembrane signal transduction pathway.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Mitsugu Maeno	Visiting Fellow	LBP, NCI
Jacob Gopas	Special Volunteer	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. In the current studies, our research effort has been focused on the further characterization of the GDP/GTP exchange factor of ras proteins.

OBJECTIVES

Our objectives are to investigate the biological role of ras proteins in cellular proliferation and differentiation with special focus on the regulatory mechanisms of the ras guanine nucleotide exchange reaction which is an important step towards initiating ras activation.

MAJOR FINDINGS

In general, the activity of a GTP-binding protein is regulated at two steps: the GDP-GTP exchange reaction ("on" step) and the GTP hydrolysis reaction ("off" step). In the case of ras protein, the regulatory mechanism of the former reaction remains unclear, while ras GAP is most likely to catalyze the latter reaction. We have recently identified and purified rGEF which accelerates the GDP-GTP exchange reaction of ras proteins. The following studies have been undertaken to investigate the biological function of rGEF:

I. Molecular cloning of cDNA encoding rGEF

To perform molecular biological studies on the physiological activities of rGEF, we have tried to isolate the cDNA encoding rGEF by using the oligonucleotide probes based on the partial amino acid sequences. Since we have not succeeded in the isolation of the cDNA by this method, we decided to carry out the immunological screening of a cDNA library by using the monoclonal antibodies against rGEF. We have obtained positive clones of the mouse hybridoma cells. Once the characterization of the clones has been completed, the antibodies will be utilized for cDNA library screening.

II. Mutational studies on the ras-rGEF interaction

In the studies of ras-rGEF interaction, the functional domain of ras proteins involved in the interaction with rGEF has been determined. We have tested various types of ras mutant proteins for their ability to respond to rGEF. In preliminary studies, the ras mutants (having a deletion at 58-63 and 64-68 amino acid residues), decreased the sensitivity to rGEF suggesting that these regions may be responsible for the interaction with rGEF. In order to further define the functional domains, we are currently making a series of ras mutants with a point mutation in these regions.

III. Studies on the involvement of rGEF activity in the control of cellular growth and differentiation

EGF, PDGF, and some lymphokines (including IL-2 and IL-3) have recently been shown to elevate the level of an active GTP-ras complex as compared to an inactive GDP-ras complex. This implies two possibilities: 1. GAP activity is suppressed or 2. the exchange factor enhances the GDP-GTP exchange reaction when the growth factors bind to their receptors. Therefore, rGEF/GAP may be regulated by the growth signal in this system. Furthermore, ras proteins could mediate signal transduction for differentiation, as well as cell growth. For example, ras protein is known to induce the differentiation of PC12 cells similar to that seen in nerve growth factor-treated cells. Our preliminary data showed that nerve growth factor increased the amount of ras-GTP complex in PC12 cells. We are now analyzing the effect of nerve growth factor on rGEF activity.

FUTURE PLANS

We will continue to study the regulatory mechanism of rGEF for ras activities and investigate the involvement of rGEF in signal transduction for cellular growth and differentiation. First, we will isolate the rGEF cDNA by screening with the monoclonal antibodies developed in our laboratory. The nature of ras-rGEF interaction will also be pursued by site-directed mutagenesis. We will extend the study on the rGEF-mediated regulation of ras activity to other biological systems, such as IL-2 and IL-6-responsive cells (cell growth) and macrophage-like differentiation of HL60 cells (cell differentiation). In the course of these studies, the neutralizing antibody against rGEF will be developed and used to dissect the signal pathway mediated by ras proteins.

PUBLICATIONS

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Manne V, Roberts D, Tobin A, O'Rourke E, De Virgilio M, Myers C, Ahmed N, Kerz B, Resh M, Kung HF, Barbacid M. Identification and preliminary characterization of farnesyl-protein transferase. Proc Natl Acad Sci USA 1990;87:7541-5.

Ono M, Kuwano M, Kung HF. Malignant transformation of mouse Balb/3T3 cells by polyoma middle T antigen requires epidermal growth factor receptor expression. Cell Growth & Differentiation 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09312-05 LBP

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.)*

Cytokine Research: Biochemical Studies of Interleukin-6 and Its Receptors

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	M. Schwabe	Visiting Associate	LBP, NCI
	G. L. Princler	Chemist	LBP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biochemical Physiology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

We have been investigating the mechanisms of action of interleukin-6 (IL-6). The research focuses on the biochemical characterization of IL-6 receptors expressed in a panel of leukemic cell lines. In addition, we are involved in studies aiming at the elucidation of the signaling pathways mediated by IL-6. We have shown that iodinated IL-6 can interact with two distinct cell surface proteins (p80 and p110), which form the basis of the high-affinity IL-6 receptor on myeloma cells. Emphasis has been directed towards establishing with some degree of certainty whether p80 and p110 are proteins encoded by different genes. We have identified a human IL-6-responsive melanoma cell line (A375-C6), which expresses significant levels of the p110 but negligible levels of the p80 IL-6 receptor. In contrast to other melanoma cells, this cell line is completely growth-inhibited by IL-6. Attempts have been made to investigate IL-6-induced biochemical changes in this cell line. Preliminary studies showed that the antiproliferative effect of IL-6 in melanoma cells (A375-C6) is associated with the induction and enhanced secretion of fibronectin, tenascin, and other extracellular matrix proteins.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Michael Schwabe	Visiting Associate	LBP, NCI
Gerald Princler	Chemist	LBP, NCI

OBJECTIVES

IL-6 has been implicated to play an essential regulatory role in a variety of immunological and neoplastic disorders. In addition, IL-6 has been suggested to be an autocrine growth factor in multiple myeloma. Since IL-6 has been implicated to play a pathogenetic role in several disease entities, such as multiple myeloma, Castleman's disease, cardiac myxoma, rheumatoid arthritis, and septic shock, the elucidation of the IL-6 receptor system and its signal transduction pathways may have clinical relevance.

MAJOR FINDINGS

The functional IL-6 receptor is currently envisioned as a two-chain receptor model comprising a ligand-binding α-chain (p80) and a non-ligand binding signal transducing β-chain (gp130). Antibodies and cDNA probes are now available for both molecules. Although the model may sufficiently explain the first step in IL-6 signal transduction, i.e. receptor binding in a particular cellular system, it cannot account for the pleiotropic actions of IL-6 (growth stimulation, growth inhibition, or induction of some cellular proteins without affecting growth) on different target tissues. In order to search for a possible explanation, we investigated the cell surface expression of the IL-6 receptor on a number of cell lines which respond differently to IL-6.

We employed iodinated and biotinylated human recombinant IL-6 to comparatively analyze the IL-6 receptor on a panel of lymphoid and non-lymphoid cell lines. By affinity-crosslinking, we detected two specific receptor bands of apparent molecular weight of 100 and 130 kD on a number of human B-cell/myeloma cell lines, such as SKW 6.4, Cess, RPMI 8226, and U266. By subtracting the molecular weight for IL-6 (20 kD), the net sizes of two IL-6 cell surface binding proteins were 80 and 110 kD (p80 and p110). Scatchard analyses indicated that all cells, which express p80 and p110, yielded curvilinear plots and gave rise to a high-affinity IL-6 receptor. On the contrary, certain monocytic cells expressed only low-affinity binding sites, as shown by Scatchard analysis, and these cells lacked the p110 protein, as determined by crosslinking. Furthermore, by treating myeloma cells with IL-6 for 24, 48, or 72 hours, we found that the p110 was no longer detectable in crosslinking and thus appeared to be down-regulated. This treatment, however, had little effect on the cell surface expression of the p80 molecule, and the curvilinear Scatchard plots reverted to linear ones with a single class of low-affinity IL-6 binding sites in the IL-6 treated population. Our results suggested that internalization of IL-6 via its receptor causes the loss of the p110 from the cell surface. The down-regulation of the p110 chain

occurred on all B-cell/myeloma cells, irrespective of their IL-6 responses (e.g. growth stimulation or upregulation of immunoglobulin synthesis).

In order to clarify the molecular identity of the p110 molecule (i.e., is p110 distinct from or related to p80 and/or gp130), we searched for cell lines which would selectively express p110 in the absence of p80. We identified the human melanoma cell line A375 which binds IL-6 with high-affinity and lacks the p80 chain, as determined by affinity-crosslinking and Northern analysis. However, we did not detect any IL-6 response in A375 cells. Among the parameters investigated are [^{3}H] thymidine incorporation, expression of HLA class I and class II antigens, integrins (α^3, α^5, α^v, β^1 and β^3 type), and the receptors for tumor necrosis factor-α (TNF-α). Subsequently, we obtained an IL-6 responsive clone of A375 cells, termed A375-C6, which has been established by others. A375-C6 cells were found to be potently growth inhibited by low doses of IL-6 (ID_{50} = 0.5 ng/ml). The C6 clone expressed low levels of p80 (as determined by crosslinking, Northern blotting, and FACS analysis), high levels of gp130 (as determined by FACS), and intermediate levels of p110 (as determined by crosslinking) compared to U266 myeloma cells. The affinity of the IL-6 receptor on A375-C6 cells, as determined by Scatchard analysis, was of an intermediate type which correlated well with the negligible amounts of the p80 and the relatively higher amounts of p110. Recently, antibodies to p80 and gp130 were made available to us, and we are now in the process of characterizing the IL-6 receptor on myeloma and melanoma cells with a combined approach of affinity-crosslinking using iodinated IL-6 and immunoprecipitation with monoclonal anti-p80 and anti-gp130 antibodies. These studies will enable us to clarify the role of the p110 molecule as part of the high-affinity IL-6 receptor.

The antiproliferative effect of IL-6 on A375-C6 cells led us to investigate IL-6-mediated biochemical changes. Interestingly, we observed that IL-6 was not cytotoxic for A375-C6 cells, but induced morphological changes (cells develop a round, refractile shape) and the apparent inability of the cells to attach to their growth surface. Since the growth of anchorage-dependent cells depend on a suitable extracellular matrix, we reasoned that the antiproliferative effect of IL-6 in this cell line could be due to changes in the extracellular matrix proteins secreted by A375-C6 cells. We demonstrated that IL-6 enhances the secretion of at least five proteins which can be detected in culture supernatants from metabolically labeled cells. We have identified two of these proteins as fibronectin and tenascin. Since both molecules belong to the family of extracellular matrix proteins, we hypothesize that the antiproliferative effect of IL-6 on A375-C6 cells could be, in part, mediated by the secretion of extracellular matrix proteins. Our preliminary results demonstrated that interfering with the synthesis and extracellular deposition of tenascin greatly affects the IL-6 response of A375-C6 cells. Further studies on the functional relationships between cytokine and extracellular matrix network will be pursued.

FUTURE PLANS

Once we have established with some degree of certainty that p110 and p80 are proteins encoded by different genes, we will prepare antibodies against p110. The availability of monoclonal antibodies against the p110 IL-6 receptor chain will enable us to investigate the structural and functional components of the

high-affinity IL-6 receptor and eventually will allow us to molecularly clone p110. The parental IL-6 unresponsive melanoma cells (A375) and its IL-6 responsive counterpart (A375-C6) can be utilized to elucidate IL-6 mediated signal transduction pathways. The involvement of G-proteins and the effect of IL-6 on protein phosphorylation will be investigated. Furthermore, the modulation of extracellular matrix proteins by IL-6 should be a useful model to study the factors required for the proliferation of anchorage-dependent cells. Also, these studies should allow us to identify a distinct set of cell surface molecules which are essential for the anchoring of melanoma cells to their growth surface. The long term goal is to develop strategies for intervention of anchorage-dependent growth of malignant cells.

PUBLICATIONS

Bristol LA, Smith MR, Bhat NK, Durum SK. IL-1 induces ornithine decarboxylase in normal T lymphocytes. J Immunol 1991;146:1509-15.

De Benedetti F, Falk L, Ruscetti FW, Colburn NH, Faltynek CR, Oppenheim JJ. Synergistic inhibition of phorbal ester-induced transformation of JB6 cells by transforming growth factor-β and retinoic acid. Cancer Res 1991;51:1158-64.

Faltynek CR, Princler GL, Schwabe M. Differential modulation of two interferon-α binding proteins. Cytokine 1991;in press.

Faltynek CR, Princler GL, Schwabe M, Shata MT, Lewis GK, Kamin-Lewis RM. Characterization of the binding of radioiodinated hybrid recombinant IFN-α A/D to murine and human lymphoid cell lines. J Interferon Res 1990;10:55-64.

Mukaida N, Gusella GL, Zachariae CCO, Matsushima K. Molecular analysis of the inhibition of interleukin-8 production by dexamethasone in a human fibrosarcoma cell line. Mol Cell Biol 1990;10:333-8.

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Mukaida N, Zachariae CCO, Gusella GL, Matsushima K. Dexamethasone inhibits the induction of monocyte chemotactic activating factor (MCAF) production by interleukin 1 or tumor necrosis factor. J Immunol 1991;in press.

Schiller JH, Bittner G, Meisner LF, Oberley TD, Norback D, Schwabe M, Faltynek CR, Raab-Traub N. Establishment and characterization of an Epstein-Barr Virus spontaneously transformed cell line derived from a hairy cell leukemia patient. Leukemia 1991;in press.

Schwabe M, Brini AT, Rubolli F, Princler GL, Faltynek CR. Detection of two distinct interleukin-6 receptors on human cell lines. In: Oppenheim JJ, ed. Molecular and Cellular Biology of Cytokines. New York: Alan R. Liss, 1990;167-72.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CM 09315-04 LBP

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.)*

Transcriptional Regulation of HIV Gene Expression

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

PI:	H. Kung	Chief	LBP, NCI
Others:	F. Ruscetti	Microbiologist	LMI, NCI
	I. Calvert	Chemist	LBP, NCI
	R. Ong	Visiting Fellow	LBP, NCI

COOPERATING UNITS *(if any)*

BCDP, PRI/DynCorp, NCI-FCRDC (M. West, J. Mikovits, Raziuddin)

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	3.0

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.)*

Monocytic cells serve as a reservoir for HIV and play an important role in the pathogenesis of AIDS. HIV-1 transcription, as well as replication, is regulated by both viral and cellular proteins. An HIV-1 infected monocytic cell line, THP-1, was established where expression is observed at various levels: 1. productive infection; 2. restricted or chronic low level expression; and 3. complete latency. Since these differences in HIV gene expression have not been molecularly defined, this system was used as a model to understand the mechanism(s) of viral latency and low level expression of the HIV genes. Transcriptional regulation depends on the sequence-specific interaction of trans-acting proteins with cis-acting DNA elements. In vitro transcription, gel mobility shift, and Southwestern assays have been utilized to identify cis-acting DNA elements and trans-acting nuclear factors which could be implicated in the restricted expression of HIV. A well-defined 30-bp DNA segment within the negative regulatory elements (NRE) of HIV-LTR strongly binds to several nuclear factors of uninfected and infected cell extracts. However, this 30-bp DNA probe (in a Southwestern blot assay) recognizes a sequence-specific DNA-binding protein present only in cells with restricted HIV expression. This protein has been molecularly cloned and characterized. The DNA-binding activity of this factor could be important in modulating HIV gene expression. Our studies could lead to the understanding of down-regulation of HIV gene expression and could serve as a potential target for AIDS therapy.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, NCI
Francis W. Ruscetti	Microbiologist	LMI, NCI
Ida S. Calvert	Chemist	LBP, NCI
Rosa Ong	Visiting Fellow	LBP, NCI

INTRODUCTION

To understand the mechanism(s) of viral latency and low level chronic expression, an HIV-1 infected monocytic THP-1 cell line was established as a model system, where initial productive HIV infection cultures become either latent or restricted in HIV expression. In cells with restricted HIV expression, we have shown that the down-regulation of the gene is associated with NF-κB binding activity (J. Mikovits et al., J. Exp. Med. 171:1705-1720, 1990). The binding complex of the fully active dimer (p65 + p50) seen in the productive nuclear extracts is not present in the nuclear extracts with restricted HIV gene expression. This loss is due to the presence of an NF-κB inhibitor, very much like the IκB, but it is localized in the nucleus rather than in the cytosolic fraction. Further studies are performed to characterize this inhibitory factor, as well as other factors involved in the negative regulation of HIV gene expression.

OBJECTIVES

The sites in the HIV long terminal repeat (LTR) that are involved in the down-regulation of the transcription of HIV genes have been reported. This region has been defined as the NRE. Our studies were initiated with two main objectives: 1. to elucidate the mechanism(s) involved in the restricted HIV expression in monocytes and 2. the role of cellular proteins recognizing this NRE region and their potential interaction with other DNA-binding proteins.

MAJOR FINDINGS

Electrophoretic mobility shift assays (EMSA) and Southwestern blot assays were primarily utilized for these studies. Using a Southwestern technique, where a 60-bp NRE region was labeled and used as the DNA probe, we have isolated and characterized a recombinant cDNA clone which encodes a novel transcription factor. This cDNA is expressed in a variety of cell types (I. Calvert et al., Gene, 1991). Another well defined 30-bp DNA segment within this NRE region strongly binds to several nuclear factors of control and HIV infected cell extracts. However, a Southwestern blot assay demonstrated a sequence-specific DNA-binding protein present only in cells with restricted expression. This protein has been molecularly cloned and sequenced. The sequences contain the typical characteristics of DNA binding proteins.

Since one objective of these studies is to identify certain transcriptional factors which are involved in HIV gene regulation, we have recently attempted to affinity purify certain factors and to study their functional roles. Using the same 30-bp NRE segment, we have made an affinity column (30-bp DNA coupled to Sepharose) and successfully purified a factor from the Hela cell nuclear extract. This factor is also a sequence-specific DNA-binding protein and acts as a transcriptional repressor molecule in our EMSA studies.

FUTURE PLANS

Our future plans include: 1. cDNAs will be subcloned into mammalian expression vectors for in vivo transfection, as well as co-infection studies; 2. attempts will be made to purify the DNA-binding proteins from the cDNA clones, as well as chronically HIV-infected monocytes; 3. antibodies against these proteins will be raised for functional studies of these DNA-binding proteins; 4. attempts will be made to characterize and clone the affinity purified Hela nuclear factor with specific binding to the NRE region; and 5. attempts will be made to isolate and identify the inhibitor(s) present in the nucleus of monocytes with restricted HIV expression and to establish its role in the negative regulation of HIV expression.

PUBLICATIONS

Calvert I, Peng ZQ, Kung HF, Raziuddin. Cloning and characterization of a novel sequence-specific DNA-binding protein recognizing the negative regulatory element (NRE) region of the HIV-1 long terminal repeat. Gene 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09316-04 LBP

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.)*

Studies of Signal Transduction in Xenopus Laevis

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	M. Maeno	Visiting Fellow	LBP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.2	1.8

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.)*

We have been using Xenopus oocytes as a model system to study the biochemical mechanisms of signal transduction pathways, especially for the functional studies of ras proteins and various BRMs. Our studies have been extended beyond oocyte maturation in order to obtain a better understanding in amphibian embryogenesis. We have established a tissue culture system in which the mesodermal cells differentiate to hemopoietic cells in early embryo of Xenopus laevis. The hemopoiesis was determined by the production of hemoglobin which was detected on the second day of the culture. In the absence of exogenous factors in tissue culture, we have shown that ventral mesodermal tissue at the late gastrula (after stage 12) has already committed itself to the differentiation for hemopoietic lineage, whereas ventral mesodermal tissue at the early gastrula (stage 10) does not differentiate to hemopoietic cells. Addition of TGF-β1 (but not TGF-β2) to the tissue culture stimulates the hemopoiesis of ventral mesodermal tissue from the early gastrula (stage 10).

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP, BRMP
Mitsugu Maeno	Visiting Fellow	LBP, BRMP

INTRODUCTION

In amphibians, it was shown that the ventral region (equivalent to the yolk sac in mammalian and avian species) of mesoderm is the primary hemopoietic differentiation site. Therefore, this mesoderm is supposed to contain multipotential hemopoietic stem cells. It is of great interest for developmental biologists to address the following questions: 1. when the commitment for hemopoietic cells occurs in this region of mesoderm; 2. what factors are necessary to determine the fate for hemopoietic cells; and 3. where the factor(s) or "determinants" are derived. The amphibian embryo is one of the most suitable model systems for these studies. The embryo is free from its mother in an early stage and is relatively large in size.

Recently, it has been shown in amphibians that mammalian FGF and TGF-β2 can induce mesoderm derivatives in explanted animal cap tissue of blastula embryos. Similar results were obtained with activin (TGF-β-like factor) which was purified from culture supernatants of the Xenopus kidney cell line, XTC-1. Therefore, it is possible that such growth factors play an important role during the tissue development on early ontogeny. In the present study, the effects of mammalian growth factor, cytokines, and oncogene products on the differentiation of hemopoietic cell lineage in Xenopus embryo will be investigated.

OBJECTIVES

The overall aim is to study the differentiation of hemopoietic cells in early embryos of Xenopus laevis. The specific objectives include: 1. establishing a tissue culture system for the ventral blood island mesoderm and 2. studying the effects of exogenous factors on the differentiation of hemopoietic cells in the tissue culture system.

MAJOR FINDINGS

1. A tissue culture system was established in which the mesodermal cells differentiate to hemopoietic cells in the early embryos of Xenopus laevis.

2. The production of hemoglobin was detected on the second day of the culture, and the total amount of hemoglobin present in the culture was the same as compared to that of an intact embryo.

3. The culture experiment of ventral mesoderms from various developmental stages showed that mesodermal tissue at the late gastrula has already committed

itself to the differentiation for hemopoietic lineage, however, mesodermal tissue at early gastrula does not.

4. TGF-β1 (not TGF-β2) stimulates differentiation of mesoderm for hemopoietic lineage.

FUTURE PLANS

Our future plans include: 1. further studies on the effects of TGF-β1 in Xenopus hemopoiesis; 2. examining the effects of other growth factors and cytokines on the development of hemopoietic cells; 3. co-culturing of ventral mesodermal cells with endoderm or other parts of the embryo; 4. establishing a dorsal mesoderm tissue culture, which contains precursor cells for hemopoietic cells in later life (after 30 days to adulthood); and 5. examining the roles of PKC, phospholipase C, and G-proteins during erythrocyte differentiation in the tissue culture system.

PUBLICATIONS

None

SUMMARY REPORT

CLINICAL RESEARCH BRANCH

October 1, 1990 through September 30, 1991

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 (BRMP) was established to facilitate early clinical trials of biological products with the potential to be anti-cancer agents. 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. The outpatient clinic of the BRMP is located just across the street from the Frederick Memorial Hospital in the Regional Cancer Therapy Center. 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 venipuncture suite, blood drawing area, a fully automated clinical laboratory, nursing station and waiting area with a library, four examining rooms, a large treatment area, a satellite pharmacy, 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. During the past year, 275 patients were entered on 22 different protocols. More than 200 other patients are currently being followed on other BRMP studies.

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 (MTD) or optimal immunomodulatory dose. Additional goals of the branch include attempts to determine the optimal method for integration of biological therapy with combination chemotherapy, and performance of studies to improve the 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 monoclonal antibodies, other biologicals, or effector cells in the management of patients with cancer. Previous CRB clinical trials 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 of the natural killer (NK) NK/LAK cell phenotype (CD56+) in the peripheral blood, that have LAK activity. These large numbers of effector cells 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 the immunomodulatory effects and

response rates associated with this regimen have included the addition of cyclophosphamide, poly ICLC, flavone acetic acid (FAA) and a monoclonal antibody specific for melanoma called R24.

Unfortunately, the addition of cyclophosphamide, poly ICLC or FAA did not result in significant changes in either immunomodulation or efficacy. However, interesting immunologic effects and significant numbers of tumor responses have been observed when this same IL-2 regimen is combined with R24. R24 recognizes a ganglioside called GD3 that is present on melanoma cells, and a subpopulation of T cells. R24 was chosen because, a) it can exert antitumor effects itself, b) it has the potential to mediate antibody-dependent cellular cytotoxicity (ADCC) with the IL-2-generated effector cells, and c) it can directly activate T cells via GD3. Twenty-one patients with metastatic melanoma have been treated to date and we have observed partial remissions in 8 of 19 evaluable patients.

In this study, the levels of peripheral blood NK and LAK activity were essentially identical in responding and nonresponding patients. Interestingly, the responders showed bursts of circulating interferon-gamma (IFNγ) in their serum after R24 and IL-2 dosing. Only minimal levels of IFNγ were detected in the serum of non-responders. These observations suggest that whether or not a patient responds to an IL-2 regimen depends on effects other than the simple presence of large numbers of NK or LAK cells; however, they do not exclude the possibility that cells with NK/LAK activity may somehow participate in the antitumor response. In another clinical IL-2 regimen, the same antibody was used, but results from this trial, though preliminary, suggest that the sequence of administration of IL-2 and antibody may be critical. R24 given before IL-2 in combination with LAK cells was associated with more toxicity than IL-2/LAK alone. When the LAK cells were omitted, response rates appear lower than when R24 is given after the IL-2. In order to develop a better understanding of the mechanisms involved, CRB studies of the clinical, immunological, and tumor histological differences between responding and non-responding patients in these trials are ongoing.

IL-2 has also been combined with interferon-alfa (IFN-α) in a phase I study employing subcutaneous outpatient administration for patients with advanced solid tumors. The maximum tolerated doses were 1.5 mu/m^2 of IL-2 daily for 5 days and 1.5 mu/m^2 daily for IFNα. After the MTD was determined, a limited phase II study in patients with renal cell carcinoma revealed 5 partial remissions in 15 treated patients. This regimen potentially could be used to maintain responses induced with higher dose regimens, or it could be combined with other agents.

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 $CD3^+$ 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 completed a phase I study of anti-CD3 in

patients with solid tumors. Dose-limiting toxicity consisted of severe headache associated with fever, photophobia and a stiff neck. The MTD was determined to be 30 mcg. 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. Animal models have demonstrated that T cells activated with anti-CD3 in vitro and then stimulated with IL-2 in vitro, or in vivo, are effective antitumor agents. In addition, these activated killer T cells (T-AK) appear to traffic specifically to tumor. Based on these findings, and the phase I study of anti-CD3 mentioned earlier, a strategy was devised to stimulate autologous lymphocytes obtained by leukapheresis ex vivo with anti-CD3 and to adoptively transfer the activated cells and treat the patients with IL-2. Immediately following IL-2, there was a fall in the lymphocyte count with a subsequent increase (up to $90,000/mm^3$) that peaked 24 to 48 hours after IL-2 was discontinued. This lymphocytosis was accompanied by typical IL-2-related toxicities (renal, hepatic, cardiovascular), but was also accompanied by a coagulopathy and a severe metabolic acidosis not seen in our previous IL-2 studies. The latter is presumed to be the result of rapid T-AK cell proliferation with concomitant glucose consumption and lactate production. Hypotension was also observed and was found to correlate with marked increases in the circulating serum nitrate levels. Production of nitric oxide has been implicated in tumor necrosis factor-mediated hypotension and an inhibitor of its action has been identified. Thus an effective method for limiting IL-2-induced hypotension may be at hand.

In contrast to studies using IL-2 alone, the cells expanded in patients receiving anti-CD3-activated cells are primarily activated T cells (CD3+, CD56-, CD25+, HLA-DR+). However, there are also increases in the non-T-cell populations of NK/LAK cell phenotype. These populations are cytotoxic for K562 and Daudi, i.e., they exhibit LAK activity. Sorting experiments indicate that most of the cytotoxic activity is in the non-T-cell subset. The single patient with melanoma that has undergone a pretreatment and posttreatment biopsy had little lymphocytic infiltrate in the original biopsy, but had a marked CD3+ CD8+ lymphocytic infiltrate in the biopsy done during therapy. There has been one partial remission identified thus far.

The recent success of 5-fluorouracil 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. To examine its effects on the immune system, we have given levamisole alone every other day for six doses, followed by a two week washout period to two groups of patients; one with metastatic disease and the other as adjuvant therapy for melanoma or renal cell carcinoma patients without evidence of disease. Following treatment with levamisole alone, patients received the same dose and schedule of levamisole in combination with IFNγ. Fifty-six patients have been treated (20 with advanced disease and 36 in the adjuvant arm). Dose-limiting toxicity was observed at the 10 mg/m^2 dose of levamisole. One partial response was noted in a patient with metastatic melanoma. NK activity was only marginally affected by levamisole alone or in combination with IFNγ, whereas serum neopterin levels were increased, but only during combined treatment.

Interleukin-1 (IL-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 had to be performed. The first phase I trial of IL-1 alfa (IL-1α) was performed at the BRMP with 28 patients receiving five different doses of IL-1α with or without indomethacin. The MTD for IL-1α alone was 0.3 mcg/kg and for IL-1α 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. An identical trial is being performed with IL-1β as the test agent. To date 20 patients have been enrolled. The side effects and hematologic effects appear nearly identical to those observed using IL-1α.

To examine the direct antitumor effects of IL-1α we have initiated a phase II study of IL-1α at 0.1 mcg/kg daily for seven days in combination with indomethacin. Thirty-two patients have been enrolled and 28 are evaluable for response. The toxicities were as expected from the phase I trial. There has been one partial response among 15 evaluable patients with nonvisceral disease and no responses in 13 patients with predominantly visceral disease.

A phase I dose escalation trial of intratumorally administered IL-1α is also in progress. Although monitoring immunological changes in the peripheral blood may provide an indication of the in vivo effects of cytokine therapy, these changes need not necessarily reflect activities within the tumors themselves. This study was designed to determine the response rates of directly administered IL-1α, and also to examine its biologic effects on the tumor cells and any lymphocytic infiltrate. To date 15 patients have been treated. Toxicity has been mild, but systemic effects (fever, chills, hypotension) have been noted following local injection. Local effects have included erythema, but no objective tumor regressions.

One of the significant findings of our phase I trial of IL-1α was the increased WBC count and the delayed increase in platelet counts found 1-2 weeks after therapy. To examine the bone marrow restorative (and BM protective properties described in animals), we are in the process of studying IL-1α for five days either before (protective) or after (restorative) an 800 mg/m^2 dose of carboplatin. Thus far, 17 patients have been enrolled, and although it is too early to evaluate the data, results indicate a beneficial effect on carboplatin-induced thrombocytopenia in individual patients.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is also being studied 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 IFNγ or IL-2. Four patients have been enrolled in this study. Other studies are investigating the ability of GM-CSF to enhance the delivery of chemotherapeutic drugs. GM-CSF is part of a protocol designed to increase the dose intensity of MOPP chemotherapy for poor prognosis patients with Hodgkin's disease.

An additional trial has been designed to take advantage of the synergistic antiproliferative effects of IFN-α and poly ICLC. To date, 63 patients with a variety of cancers have been enrolled. The addition of poly ICLC at a variety of doses (0.03-1.0 mg/m^2) did not alter the toxicity profile of IFN. Dose-

limiting toxicity was observed with IFN at 10 mu/m^2. Three patients among 52 evaluable patients have had partial responses. A unique combination of agents (5FU, leucovorin, azidothymidine (AZT) and persantine; FLAP) has been used in patients with melanoma, colorectal and renal cell cancer. A phase I portion of the study determined that AZT at 200 mg four times a day was tolerable. A phase II portion of the study noted no responses in 14 patients with melanoma and renal cell carcinoma, respectively. There has been one partial response in seven patients with colorectal cancer; this portion of the study remains open for additional accrual.

One of the crucial unanswered questions in the immunologically-based therapy of human cancers is whether naturally arising human tumors bear cell surface antigens that can be targeted by specific immune effector cells in a clinically meaningful way. A randomized trial in patients with stage B_2 or C colon cancer showed that autologous tumors prepared from primary lesions and given with Bacille Calmette-Guerin (BCG) induced specific delayed type hypersensitivity (DTH) responses that were associated with improved survival. The CRB is performing a trial in patients with kidney cancer to see if patients immunized with their own cancers and BCG will react more often and more strongly to their tumor if they are also given IL-2. Eight patients have been enrolled on this protocol.

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-α or after completion of therapy with alternating cycles of IFN-α and deoxycoformycin. Of the former, 32 patients continue to receive IFN without interruption for a median of 72 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-α2a suggesting that alternative non-cross reactive species of IFN may be used in these patients. Further follow-up of patients with neutralizing antibodies, but 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 later interferon-related toxicities but two patients developed an absolute erythrocytosis late in the course of therapy leading to discontinuation of IFN in one. Statistical analysis of patients' hemoglobin, platelet counts, and granulocyte counts revealed a significant improvement in the platelet and granulocyte counts even after 18 months of IFN therapy.

Fifteen patients continue to be followed for a median of 49 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. Statistical analysis indicates that in 13 of 14 patients the number of CD4+ T cells is increasing significantly with time off treatment. However, it is incorrect to

say that the patients T-cell profile is normal, because only 4 of the 14 evaluable patients have CD4+ T-cell percentages that consistently fall in the normal range.

The CRB also maintains clinical protocols for the management of patients with non-Hodgkin's lymphomas and Hodgkin's disease. Patients with aggressive lymphomas are 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 56 evaluable patients entered on study of whom 54 (96%) achieved a complete remission. There has been one relapse and only three deaths. Advanced stage patients are treated with short course ProMACE-CytaBOM, a regimen 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 49 evaluable patients and 42 have achieved complete remissions (86%). There have been 7 relapses (17%). 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 six patients entered have achieved a complete remission. There have been two relapses; one patient is currently disease-free after high-dose chemotherapy and autologous bone marrow transplant and the second is undergoing similar treatment.

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. After treating 123 patients, there are no significant differences in disease-free or overall survival; however, there are differences in complete response rates between the two groups with 74% of patients randomized to intensive therapy achieving a complete remission compared to 40% 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 early stage Hodgkin's disease are randomized to MOPP chemotherapy or radiation. Of the 55 evaluable patients randomized to MOPP, 53 (96%) achieved a complete response; 7 relapsed. Fifty of 52 patients treated with radiation achieved a CR (96%); 19 patients (38%) 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. 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. To date 10 of the 12 evaluable patients receiving dose intense MOPP have achieved a complete remission and there has been only one relapse.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09291-06 CRB

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.)*
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:	J. W. Smith II	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
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

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

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

We administered deoxycoformycin (dCF) and interferon-alfa (IFN-α) 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 IFN-α resistant patients were nearly uniformally responsive to second line dCF. As there was some overlapping toxicity, 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 IFN-α 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 IFN-α 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 49 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.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	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
Stephen P. Creekmore	Chief	BRB, NCI

OBJECTIVES

1. To determine the qualitative and quantitative toxicities of 2'-deoxycoformycin (dCF) and interferon alfa (IFN-α) 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 dCF and IFN-α.

4. To determine the biochemical consequences of administering dCF 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 IFN-α or dCF. Patients were evaluated in the outpatient clinic of the Clinical Research Branch and were hydrated and given 4 mg/m^2 of dCF intravenously as a one-half hour infusion. After infusion of the dCF, 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 dCF 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 IFN-α at a dose of 3 million units/m^2 is administered. This two-month cycle of dCF 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 two years, and yearly thereafter. For patients responding well to therapy and tolerating the therapy well, some of the dCF 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. 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 49 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 overdose of sleeping pills 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 dCF 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 IFN-α. Patients receiving deoxycoformycin had rapid and very marked drops in total lymphocyte counts and in total numbers of $CD4^+$ and $CD8^+$ lymphocytes. Although most patients had normal $CD4^+$ cell levels and normal T4 to T8 ratios prior to beginning therapy, all patients had decreases in $CD4^+$ lymphocyte numbers to less than 200/mm^3 shortly after the first course of dCF therapy.

$CD4^+$ cell numbers remained depressed for the duration of therapy and remained depressed after completion of dCF. The duration of suppression of $CD4^+$ cell counts has been followed closely in these patients. As of August 1991, eight

patients' (53%) $CD4^+$ T-cell numbers returned to normal by a mean of 11 months (range 3-27 months) from the end of therapy and have remained in the normal range after recovering. However, six patients continue to have abnormally low $CD4^+$ T-cell counts a mean of 44 months (range 35-47 months) from the completion of therapy to the time of last evaluation. Based on our statistical analysis, 8 of the 14 evaluable patients have experienced significant normalization of their peripheral $CD4^+$ T-cells during and after therapy. Five of the remaining 6 patients had a significant improvement in $CD4^+$ T-cell values with time after completion of treatment.

Thus, while there is sufficient statistical evidence to conclude that the number of $CD4^+$ T cells increases significantly with time off treatment in 13 of 14 hairy cell leukemia patients treated with dCF and IFN-α, it is incorrect to interpret the data as indicating that the peripheral blood T-cell phenotype is normal. Only 4 of the 14 patients have $CD4^+$ T-cell percentages that consistently fall in the normal range; four have 25-35% $CD4^+$ T cells and six have <25% $CD4^+$ T cells.

No secondary malignancies or opportunistic infections have developed in any patient. A total of seven cases of dermatomal Herpes zoster have been noted.

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 dCF suggested that this drug was capable of inducing a high rate of durable complete remissions, 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 IFN-α and dCF will lead to substantially greater degrees of initial response than expected with dCF alone. However, only by comparison of the duration of response in our patients to the duration of response in patients receiving dCF 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 dCF alone. Clearly, this regimen is reasonably well tolerated and brings about peripheral blood and marrow responses much more rapidly than does IFN-α.

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 dCF 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 dCF. Although CD4 T-cell numbers appear to be recovering over time, the peripheral T-cell profile of many patients remains abnormal. Prolonged follow-up of these patients will be necessary to determine the late clinical consequences of this immune suppression. In trying to decide between dCF or IFN treatment for hairy cell leukemia, immune suppression by dCF and its consequences should 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

Steis RG, Urba WJ, Kopp WC, Alvord WG, Smith II JW, Longo DL. Kinetics of recovery of CD4+ T cells in the peripheral blood of deoxycoformycin-treated patients. J Natl Cancer Inst 83:1678-9, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09305-05 CRB

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.)*
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:	D. L. Longo	Associate Director	OAD, BRMP, NCI
Others:	J. W. Smith II	Medical Officer	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (N. Engler); 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:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

This study 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 six 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 five 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

Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	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 interferon-alpha (IFN-α).

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 IFN-α are eligible for this study if they have progressive disease during the course of IFN administration. Patients with hairy cell leukemia are also eligible for the study if the side effects of IFN-α are intolerable. Patients with T-gamma lymphoproliferative disorder are eligible for this study if they have not responded to three consecutive months of IFN-α 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

Six patients with interferon-resistant hairy cell leukemia have been treated and three partial responses were obtained. Two patients did not respond and one patient died of refractory thrombocytopenia and gastrointestinal bleeding after two doses of dCF. This rate of response (50%) 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 and one patient with T-gamma lymphoproliferative disease and coronary artery disease who was ineligible for interferon therapy 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. The most recent patient with coronary artery disease has been on therapy for three months and thus far has had complete resolution of his requirement for packed red blood cell transfusions.

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 CLL. 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 09306-05 CRB

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.)*
Phase Ib Trial of Intraperitoneal GM-CSF

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

PI:	B. L. Gause	Medical Officer	CRB, NCI
Others:	J. E. Janik	Expert	CRB, NCI
	J. W. Smith II	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller)

LAB/BRANCH
Clinical Research Branch

SECTION

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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.)*

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 peripheral blood monocytes 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 this can be accomplished in humans, the hypothesis that monocytes and macrophages can bring about tumor responses in humans can be tested. In three separate parts of this study patients will receive either GM-CSF alone, GM-CSF with interferon-gamma (IFN-γ), or GM-CSF with interleukin-2 (IL-2). All drugs will be administered intraperitoneally. Four patients have been enrolled and treated so far. We have seen substantial increases in the number of monocytes and granulocytes in the peritoneal fluid of three patients. No tumor responses have been observed. Thus, monocytes can be recruited to the peritoneal cavity, but additional patients will be required before activation of monocytes in vivo and antitumor activities in vivo can be assessed.

PROJECT DESCRIPTION

PERSONNEL

Barry L. Gause	Medical Officer	CRB, NCI
John E. Janik	Expert	CRB, NCI
John W. Smith II	Medical Officer	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 IFN-γ or with IL-2.

2. To determine the effects of intraperitoneal injections of rHuGM-CSF alone or with IFN-γ 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 IFN-γ 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 receive 5 additional 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 IFN-γ 5 days per week for 3 consecutive weeks. The dose of GM-CSF will be escalated in groups of 3 patients but IFN-γ will be given at a constant dose of .05 mg/m^2. 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 IFN-γ, 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 computed tomography scans or peritoneoscopy.

MAJOR FINDINGS

To date only 4 patients have been entered on study. Three patients completed all ten days of intraperitoneal GM-CSF in Part 1 of the study. Two of these patients were found to have a marked increase in the cell counts with significant increases in the number of macrophages and monocytes in the peritoneal cavity. The only toxicities noted during the course of administration of GM-CSF were low-grade fever and palmar erythema. The third patient completed all three treatment phases. This patient had minimal increases in neutrophils and no evidence of increase in intraperitoneal monocytes or macrophages. At the two month follow-up evaluation, the patient's disease was stable and she is being followed off therapy. The fourth 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 intraperitoneal 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 in some patients. 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 IFN-γ.

SIGNIFICANCE

A major potential limitation of adoptive immunotherapy of cancer is suboptimal trafficking of ex vivo activated cells following their intravenous administration. Monocytes activated ex vivo with IFN-γ 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 IFN-γ or IL-2.

PROPOSED COURSE

This study remains open for further patient entry. Amendments to the protocol are being made in an attempt to increase patient accrual.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09308-05 CRB

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.)*
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:	J. W. Smith II	Medical Officer	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

See next page

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller)

LAB/BRANCH
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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 receiving 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, 40 partial remissions, 2 minor responses, 9 patients with stable disease and only 1 patient with disease progression. In the last year, one of the patients with a minor response has now obtained a partial remission. Thirty-two patients continue to receive interferon without interruption with a median duration of continuous interferon treatment of 6.5 years. Twenty-one patients discontinued interferon for a variety of reasons, the most common being the development of acquired interferon resistance in association with interferon antibodies. The resistance to interferon was manifested early, in the first 18 months of treatment. An important finding in this study is the continued slow, but significant, hematologic improvement in absolute granulocyte and platelet counts beyond 18 months of therapy, thereby indicating that prolonged treatment results in continued benefit rather than the production of antibodies with subsequent development of interferon resistance. Although it is clear from this study that hairy cell leukemia can be controlled in the long-term with interferon, longer follow-up will be necessary to determine if continuous therapy with interferon is better than intermittent therapy.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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
Barry L. Gause	Medical Officer	CRB, NCI
Jon T. Holmlund	Medical Officer	CRB, NCI
Brendan D. Curti	Staff Fellow	CRB, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI

OBJECTIVES

To evaluate the safety and efficacy of recombinant interferon-alfa (IFN-α) in patients with hairy cell leukemia.

METHODS EMPLOYED

Eligible patients 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 IFN-α 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 IFN-α in an attempt to control their disease. If dose-limiting toxicity develops 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 multiform, 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. Patients best response to therapy included one complete remission, 40 partial remissions, two minor responses, nine 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 was disease progression following initial responsiveness. This acquired interferon resistance occurred in the first 18 months of treatment and 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 IFN-α2a; 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 IFN-α2a. We only had one patient who had a neutralizing antibody against IFN-α2a 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 IFN-α2a 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 IFN-α2a. Further, there was no induction of neopterin or 2', 5'oligoadenylate synthetase activity following injection of IFN-α2a. 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 IFN-α2a. The in-vivo effects thus mirror the in-vitro antibody specificity. The fact that patients with neutralizing antibodies still remain responsive to interferon 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 IFN-α; 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.

The patients' serial hemoglobin, platelet counts and granulocyte counts were analyzed to determine if there was any improvement in these parameters that would prolong treatment beyond 18 months. Statistical analysis revealed a significant improvement in the platelet count and absolute granulocyte count with continued therapy beyond 18 months. The hemoglobin level remained the same during this period. An analysis was performed to determine if this phenomenon occurred in patients who never had anti-interferon antibodies detectable in the serum. It was established that this group of patients showed significant improvement in the platelet count and absolute granulocyte count beyond 18 months of therapy similar to the group of patients who had evidence of detectable anti-interferon antibodies.

The overall survival for all 56 patients is 82%. Of the 10 deaths, three were due to progressive disease, one to infection, three to second malignancies, and three from cardiovascular causes.

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 in this study, or perhaps 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 among 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, Urba WJ, Venzon DJ, Longo DL, Barney R, Evans LM, Itri LM, Ewel CH. Loss of interferon antibodies during prolonged continuous interferon-α2a therapy in hairy cell leukemia. Blood 1991;77:792-798.

Smith II JW, Longo DL, Urba WJ, Clark JW, Watson T, Beveridge J, Conlon KC, Sznol M, Creekmore SP, Alvord WG, Lawrence JB, Steis RG. Prolonged, continuous treatment of hairy cell leukemia patients with recombinant interferon-α2a. Blood 1991;78:1664-71.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09329-03 CRB

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.)*
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:	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./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol); Medicine Branch, NCI, Bethesda, MD (E. Reed)

LAB/BRANCH
Clinical Research Branch

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NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	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.)*

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 carboplatin. A previous study with high-dose carboplatin demonstrated a response rate of 27% in patients with advanced ovarian carcinoma. Therapy was associated with severe and prolonged myelosuppression. In this trial, carboplatin 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 carboplatin. This study was conducted jointly with the Medicine Branch of the National Cancer Institute. Eight patients have been treated at the Biological Response Modifiers Program (BRMP). All patients experienced significant myelosuppression following high-dose carboplatin 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. In seven evaluable patients treated at the BRMP, there were three partial and one complete responses.

PROJECT DESCRIPTION

PERSONNEL

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
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 carboplatin (800 mg/m^2) in patients with refractory ovarian carcinoma.

2. To determine if the dose intensity and/or cumulative dosage of carboplatin may be increased in association with rGM-CSF administration.

3. To compare the response rate of high-dose carboplatin with rGM-CSF to historical controls treated without rGM-CSF.

4. To evaluate the toxic effects of high-dose carboplatin and rGM-CSF.

METHODS EMPLOYED

Patients entering this trial must have a histologic diagnosis of an epithelial ovarian carcinoma refractory to prior therapy. Prior therapy may include cisplatin unless doses of greater than or equal to 150 mg/m^2 were administered without response. Patients must also have adequate bone marrow, hepatic and renal function with serum creatinine levels less than 1.5 mg/dl, white blood count greater than 3,000 per cubic millimeter, 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 given carboplatin at a starting dose of 800 mg/m^2 every 35 days. rGM-CSF is given as a subcutaneous injection starting 72 hours after carboplatin 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 included 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 carboplatin and GM-CSF therapy.

MAJOR FINDINGS

Eight patients have been admitted to this study at the BRMP. Three 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 was thrombocytopenia, which in some patients has been refractory to random donor platelet administration and to HLA matched platelets. All of the patients experienced Grade IV platelet toxicity with platelet counts below 20,000. Several of the patients experienced bleeding complications including petechiae, hematuria, epistaxis and gastrointestinal hemorrhage. Fever was universally seen in association with rGM-CSF administration, and in all but two 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 carboplatin 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 carboplatin. 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 carboplatin to this group of patients.

PROPOSED PLAN

This study is closed to accrual and no further patients will be entered.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09331-03 CRB

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.)*
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	Medical Officer	CRB, NCI
	R. G. Fenton	Expert	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*
Frederick Memorial Hospital (C. Donovan); Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba, J. Beveridge); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol)

LAB/BRANCH
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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 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	Medical Officer	CRB, NCI
Robert G. Fenton	Expert	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. Further analysis of immune modulatory effects suggests a possible dose-related enhancement of NK activity with increasing doses of Poly ICLC up to 1 mcg, over that of IL-2 alone. The clinical significance of this finding is uncertain, since no antitumor responses were seen.

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. The laboratory analysis of the immune effects of Poly ICLC in these patients suggests a dose-dependent enhancement of NK activity, in combination with IL-2, over the effects of IL-2 alone. The clinical significance of this finding is uncertain, especially since no antitumor responses were seen. 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 09332-03 CRB

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.)*
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	Medical Officer	CRB, NCI
Others:	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
	See next page		

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller); 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:
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.)*

Interleukin-1 alpha (IL-1α) possesses antiproliferative, immunostimulatory, anti-infection, myeloprotective, and myelorestorative properties that could be beneficial in cancer treatment. In this phase I trial, IL-1α was administered IV over 15 minutes daily for 7 days to patients with advanced solid malignancies. The maximum tolerated dose (MTD) of IL-1α alone was 0.3 μg/kg. A second group of patients received indomethacin plus IL-1α based on preclinical studies indicating that indomethacin could abrogate IL-1α-induced hypotension; however, the maximum tolerated dose of IL-1α plus indomethacin, 0.1 μg/kg, was lower than IL-1α alone. Fever, chills, headache, nausea, vomiting, and myalgia were commonly observed but were not dose-limiting. Hypotension resulted from a marked decrease in systemic vascular resistance and required pressors at 0.3 and 1.0 μg/kg IL-1α. Dose-limiting toxicities included hypotension, myocardial infarction, confusion, and renal insufficiency. IL-1α treatment caused a significant, dose-related increase in total white blood count (mainly segmented neutrophils and neutrophilic bands). Bone marrow cellularity increased due to enhanced numbers of relatively mature myeloid cells and megakaryocytes. Platelet counts declined during therapy but were significantly elevated above baseline 1-2 weeks after the end of treatment; this may have been due to IL-6 that was shown to be induced by IL-1α treatment. Significant increases in triglycerides, cortisol, C-reactive protein, thyroid stimulating hormone and decreases in cholesterol, testosterone and protein-C were observed with treatment. Pharmacokinetic studies indicated IL-1α to have a short half-life of 10-15 minutes. We conclude that at doses of IL-1α that can be safely given to cancer patients, significant, potentially beneficial hematopoietic effects occur.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	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
Barry L. Gause	Medical Officer	CRB, NCI
Brendan D. Curti	Staff Fellow	CRB, NCI
Jon T. Holmlund	Medical Officer	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 mg every 8 hours plus IL-1 alpha beginning with a dose two levels 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, three 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 occurred 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°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, albumin and serum potassium 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 up to 7-fold 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 an average of 1.7-fold above baseline 1-2 weeks later in five patients treated with IL-1 alpha plus indomethacin. Bone marrow aspirates obtained before treatment and one day after completing seven days of treatment with IL-1 alpha showed a marked increase in cellularity and an increase in the M/E ratio. In addition, there was an increase in the number of megakaryocytes in proportion to the increase in cellularity in patients treated with doses of IL-1 $\geq$ 0.1 μg/kg. 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 GM-CSF 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 highest dose level. Pharmacokinetic data indicated a short 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 CD56+ cells.

Significant increase in triglycerides, cortisol, C-reactive protein, and thyroid stimulating hormone were observed with treatment, while decreases in cholesterol, testosterone, and protein-C occurred during therapy.

SIGNIFICANCE

This phase I study of IL-1 alpha demonstrated important biological activity at doses that are well tolerated in humans. There were a wide range of toxicities from this agent involving the cardiovascular, renal, gastrointestinal, and central nervous systems. Hypotension, myocardial infarction, 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 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 or 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 caused significant hematologic effects including a marked increase in the peripheral WBC, platelet count, and an increase in the relatively mature myeloid elements and the number of megakaryocytes in the bone marrow.

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 are nearing completion which are investigating the antitumor activity of IL-1α in patients with metastatic melanoma and the ability of IL-1α to prevent or reduce bone marrow suppression from high dose chemotherapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09335-03 CRB

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.)*

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	Medical Officer	CRB, NCI
	K. C. Conlon	Senior Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc./DynCorp, 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:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

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.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John W. Smith II	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 09336-03 CRB

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.)*
Short-Course ProMACE-CytaBOM for Stages II-IV Diffuse Aggressive Lymphoma

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

PI:	D. L. Longo	Associate Director	OAD, BRMP, NCI
Others:	BRMP Staff		BRMP, NCI
	P. L. Duffey	Research Specialist	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

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

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

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 and stages III and IV follicular mixed lymphoma. The treatment consists of eight 2-week cycles, for a total of sixteen weeks. A dose escalation schema has been added and most patients are able to be escalated to dose level three. To date, there have been 49 evaluable patients entered on study: 42 have achieved a complete response (86%). There have been 7 relapses (17%), all following short complete remissions. 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 and advanced stage follicular mixed lymphoma, 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. Doses of cyclophosphamide, doxorubicin and etoposide may be escalated to predetermined dose levels 1 and 2 if hematologic toxicity permits. Patients whose blood counts allow are escalated to dose level 2 and dose level 3. 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

Fifty-three patients have been entered on this study. Four are too early to evaluate. Of the 49 evaluable patients, 42 (86%) have achieved a complete response. Seven patients (17%) have relapsed from complete remission. Four of the six patients who were induction failures have died with progressive disease despite aggressive retreatment. One induction failure is currently undergoing autologous bone marrow transplantation (ABMT) for salvage. One induction failure, who had microscopic indolent lymphoma found at laparotomy, has undergone abdominal radiation therapy and continues in complete remission. All seven relapses have occurred after complete remissions of short duration in previously involved sites: one patient remains in a second complete remission after ABMT; one is alive with disease. Four relapsers have died: one died with progressive disease during retreatment by a local physician; one achieved a second complete remission with ABMT, relapsed in a new extranodal site, then underwent allogeneic BMT but died from pneumonia/sepsis without evidence of disease; one achieved a second complete remission after ABMT, but relapsed again, received radiation, EPOCH, and died with progressive disease; and one achieved a second complete remission with ProMACE, relapsed and died with progressive disease.

SIGNIFICANCE

Although the results of this regimen are preliminary and only small numbers of patients with a relatively short follow-up time have been treated, 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 tolerable toxicity.

PROPOSED COURSE

This study remains open and continues to accrue patients. The replacement study is being written.

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 09337-03 CRB

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.)*
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./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

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 rates and survival. In order to improve the efficacy of MOPP therapy in this group of patients, the dose-intensity of all drugs has been increased and GM-CSF is given to ameliorate the severe myelotoxic effects of the increased drug doses. To date, 10 of 12 evaluable patients have achieved a complete response. One patient has relapsed. One patient who was an induction failure has since undergone an allogeneic bone marrow transplant and achieved a complete response. The other induction failure is currently undergoing salvage therapy and is too early to evaluate. The one relapsed patient underwent ABMT, but relapsed following a short CR. He is currently receiving other therapy and is too early to evaluate. 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, 14 patients have been enrolled in the study and 12 are evaluable. One patient is too early to evaluate and one patient dropped out in cycle 2 for reasons unrelated to treatment. Of the 12 evaluable patients, 10 have achieved a complete response. One patient has relapsed; none has died. The patient who was an induction failure has since undergone an allogeneic bone marrow transplant and achieved a complete response. The other induction failure is currently undergoing salvage therapy and is too early to evaluate. The one relapsed patient underwent ABMT, but relapsed following a short CR. He is now too early to evaluate for response to current therapy. 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 09339-03 CRB

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.)*
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:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

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 55 evaluable patients randomized to MOPP, 53 (96%) achieved a complete response; 7 relapsed. Fifty of the fifty-two randomized radiation-treated patients (96%) achieved a complete response: 19 patients (38%) relapsed. Four MOPP-treated patients (7%) and eleven (21%) 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 than 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 52 patients have been randomized to radiation; 50 (96%) have achieved a complete response. Nineteen patients (38%) have relapsed from CR. Among the 19 patients relapsing from a radiation therapy-induced CR, 1 received MOPP-ABVD chemotherapy and failed to obtain a second complete response. Eighteen patients

were treated with MOPP; two are too early to evaluate; 11 (73%) achieved a complete remission and 8 (53%) continue in second remission for periods ranging from 2-12 years. One patient died in second remission. Two patients relapsed from second remission and one achieved a third remission after high dose therapy and bone marrow transplantation, but relapsed again and died. There have been a total of 11 deaths (21%): 8 with progressive disease and 3 with no evidence of disease (one sepsis during MOPP reinduction, 1 AIDS, 1 pneumonia).

Randomized to MOPP:

Fifty-six 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-three of 55 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 (6+ 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 one 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.

PUBLICATION

Longo DL, Glatstein E, Duffey PL, Young RC, Hubbard SM, Urba WJ, Wesley MN, Raubitschek A, Jaffe ES, Wiernik PH, DeVita, Jr, VT. Radiation therapy versus combination chemotherapy in the treatment of early-stage Hodgkin's disease: Seven-year results of a prospective randomized trial. J Clin Oncol 1991; 9:906-17.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09340-03 CRB

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.)*
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
	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:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

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

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

This study was designed 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 74% of patients randomized to intensive therapy achieving a complete response compared to a CR rate of 40% in those treated after a period of observation. The median follow-up time is six years, shorter than the median survival (10 years) of patients with these diagnoses. Continued follow-up may show a benefit for a subset of patients.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, 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 156 patients entered on study with 132 randomized. Ten patients are not evaluable because each did not receive the treatment to which they were randomized: Four on WW and six on intensive therapy. Median follow-up is over 6 years.

Randomized to Intensive Therapy: Of the 61 evaluable patients on this arm, 45 achieved a CR (74%). There have been 15 relapses (33%) and 19 (30%) have died. Twenty-nine patients (47%) continue alive and free of disease on this arm. There have been no unexpected toxicities with this therapy.

Randomized to Watch and Wait: There are 62 evaluable patients on this arm. Twenty-nine patients remain on WW and are alive with lymphoma. Thirty-three patients (53%) have crossed-over to intensive therapy. Two are too early to evaluate and 12/30 (40%) patients have achieved a complete response; four (33%) have relapsed from CR. Seventeen patients (27%) have died. Seven 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-four patients who were not suitable for randomization were treated with intensive therapy at diagnosis. One continues on therapy and is too early to evaluate. Of the 23 patients evaluable for response, 13 (57%) have achieved a complete response. Four of 13 (31%) have relapsed and 8 of the 23 (35%) 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 just over 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. The replacement study is currently being written.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09341-03 CRB

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.)*
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./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [x] (b) Human tissues
- [ ] (c) Neither

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

This protocol was designed to improve the prognosis of patients with all grades of angiocentric immunoproliferative lesions. Six patients have been entered on study. All six patients have achieved a complete response and 2 have relapsed. One relapsed patient has since undergone bone marrow transplantation and remains in second CR lasting 1.5+ years. The second relapsed patient is currently undergoing ABMT and is too early to evaluate. No patient has died. More patients and longer follow-up are necessary to draw any conclusions.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

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 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.

Six patients with Grade III AIL's have been entered to this study. All six patients have achieved a complete response and 2 have relapsed. One relapsed patient has since undergone bone marrow transplantation and remains in second CR. The other relapsed patient is undergoing ABMT and is too early to evaluate. No patient has died. 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

PROJECT NUMBER: Z01 CM 09342-03 CRB

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.)*
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:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

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

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

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 56 evaluable patients entered on study of whom 54 (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.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Patricia L. Duffey	Research Specialist	OAD, BRMP, NCI
BRMP Staff		BRMP, NCI

OBJECTIVE

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-eight patients have been entered on this study. Two are currently receiving chemotherapy and are too early to evaluate, two patients completed less than 2 cycles of chemotherapy for reasons unrelated to treatment, but are counted as non-responders. Fifty-four of fifty-six (96%) patients achieved a complete response and only one has relapsed. Three patients died; 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 one relapsed patient underwent ABMT, achieved a second CR, but relapsed. She is alive with lymphoma undergoing other therapy. 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.

SIGNIFICANCE

With a complete response rate of 96% and a relapse rate of 2% after a median follow-up of over 5 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 09350-02 CRB

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.)*
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	Medical Officer	CRB, NCI
Others:	S. P. Creekmore	Chief	BRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, 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./DynCorp, 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
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SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	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 purpose of this protocol was to examine the immunologic effects of levamisole using modern assay techniques in order to determine if an alternate dose and schedule of levamisole administration would produce maximal immunomodulation. Levamisole was also administered in combination with interferon-gamma, a cytokine whose immunomodulatory properties have been well defined in two previous trials conducted at the Biological Response Modifiers Program.

Levamisole was administered orally every other day at one of four different dose levels. Two groups of patients were eligible for entry on this trial. One group had advanced metastatic disease and the second group (adjuvant group) was treated after surgical therapy to render the patient disease free. Levamisole alone was administered every other day for a total of six doses, after a ten-day rest the same dose of levamisole was given in combination with interferon-gamma at a dose of 0.1 mg/m sq subcutaneously every other day on the same days as the levamisole.

Dose-limiting toxicity was observed at the 10 mg/kg dose of levamisole in both groups of patients. The maximum-tolerated dose was defined as 5 mg/kg every other day. At this dose level, treatment was well tolerated with levamisole alone and with levamisole plus interferon gamma.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Medical Officer	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, 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 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, computerized tomography (CT) of the brain, and three baseline immune parameters. 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 every other day for two weeks (a total of six doses). The patients then had ten days of rest after which they resumed treatment with the same dose of levamisole with IFN-γ 0.1 mg/m^2 subcutaneously every other day. Patients with advanced cancer were treated with the combination of the two agents for a total of one month. Responding patients received additional cycles. 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 of levamisole until the MTD was defined. The doses of levamisole 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 (LAK) cell activity, serum soluble interleukin-2 receptor level, serum IFN-γ level, serum neopterin level, monocyte hydrogen peroxide production, and cell surface expression of Fc receptor and class II molecules.

MAJOR FINDINGS

This protocol enrolled 56 patients, 20 patients with advanced disease and 36 patients in the adjuvant setting. Two patients in the advanced setting are not eligible for toxicity evaluation only of levamisole alone due to early removal because of disease progression. One of the patients in the adjuvant group discontinued therapy after the levamisole alone portion of the protocol and was replaced. There were 10 men and 10 women in the advanced disease group and 26 men and 10 women in the adjuvant group. Eleven patients with melanoma, four with colon cancer, two each with breast and kidney cancer and one patient with a malignant chordoma comprised the advanced disease group. Thirty-three patients with melanoma and three with renal cell cancer comprised the adjuvant group. The median age was 59 (range 36-75) for the advanced disease patients and 42 (range 19-73) for the adjuvant group.

Dose-limiting toxicity was observed in three of five patients treated at the 10 mg/kg dose in the adjuvant setting and two of five patients with advanced disease. A syndrome of fever, chills, diffuse skin erythema, moderate to severe headaches without meningismus and severe nausea and vomiting resulting in dehydration occurred in three women, two treated in the adjuvant setting and one with advanced disease. Two of these women were rechallenged with the same dose of levamisole and had an immediate recurrence of these symptoms whereas this toxicity occurred after four or five doses during the initial exposure to the drug. One of these three patients was subsequently treated with 2.5 mg/kg of Levamisole and tolerated this dose without significant toxicity. In two of these patients, neopterin levels were significantly elevated and low levels of IFN-γ were detected. Increases in neopterin levels were occasionally detected in other patients at lower dose levels. This would indicate that the toxicity these women experienced may not have been an allergic reaction to the drug, but was perhaps immunologically mediated. One additional patient in the adjuvant setting had dose-limiting nausea and vomiting with levamisole at 10 mg/kg when it was combined with IFN-γ, despite tolerating the levamisole alone relatively well. He was able to tolerate a total dose of 10 mg/kg every other day when the dose was

split into 5 mg/kg portions. One patient with metastatic melanoma in the advanced disease setting had Grade 3 hypertension that prevented further therapy.

At the lower doses of levamisole (1 mg/kg and 2.5 mg/kg), toxicity was mild, Grade 1 or 2 in all cases. Central nervous system toxicity was frequently observed and consisted primarily of anxiety, depression, light headedness, difficulty concentrating and insomnia. Insomnia was more frequent during the period of combined levamisole and IFN-γ therapy. At the 5 mg/kg dose level, the toxicity was similar but increased in frequency and grade. One patient had Grade 3 headache during the levamisole alone portion of the protocol. A lumbar puncture was performed and showed no abnormalities. The patient was rechallenged without recurrence of these severe headaches. Three patients experienced Grade 3 or 4 CNS disfunction. Two patients had therapy discontinued because of this toxicity. One patient had suicidal ideation during the combined Levamisole/γ-IFN therapy and was removed from study. His thought disorder resolved with discontinuation of therapy. Another patient had headache, photophobia, and meningismus during combined therapy. CT and magnetic resonance imaging scans of the head were negative as were the results of a lumbar puncture. Her toxicity resolved with discontinuation of therapy. The final patient experienced an episode of confusion, disorientation, and was involved in an automobile accident several days after completing the Levamisole alone portion of the protocol. Evaluation with CT of the head and electro-encephalogram was negative. A lumbar puncture performed 10 days after completing the initial Levamisole therapy showed an elevated total protein of 65 with a white blood cell count of 40 per cubic millimeter, 100% lymphocytes. Therapy was resumed with Levamisole and γ-IFN without recurrence of these symptoms. Two additional lumbar punctures performed in this patient were negative showing a normal protein and white blood cell count.

Granulocytopenia is reported to occur in 3-5% of the patients treated with levamisole and is associated with a maturation arrest of bone marrow granulocyte precursors. Granulocytopenia was frequently observed in this study with 25% of the patients experiencing Grade II, 4% Grade III and 4% Grade IV neutropenia. In all cases, granulocytopenia occurred during the combined therapy portion of the protocol. In all cases but one, this toxicity was probably related to IFN-γ's effect. One patient, however, had an absolute granulocyte count of 0 with a maturation arrest of granulocyte precursors at the myelocyte stage in the bone marrow, consistent with the described marrow toxicity of levamisole. In all cases, the granulocyte count returned to normal following discontinuation of therapy. It would appear that IFN-γ does not accentuate the marrow toxicity of Levamisole with only 1 of 49 patients treated with both levamisole and IFN-γ experiencing this severe toxicity.

One partial response was seen in a patient with malignant melanoma. This patient had received previous therapy with IFNα and IL-2 and LAK cell therapy. The partial response occurred after 8 months of combined therapy and she continues to respond at 19+ months after initiation of therapy. A second patient with melanoma achieved a minor response of 8 months duration. All other patients had stable or progressive disease. Thirty-six patients were treated in the adjuvant setting. One patient discontinued therapy after the levamisole alone portion of the protocol and was removed from study at her request. Twenty-four of the patients at last follow-up had relapsed and 11 of the patients remained disease

free with 0 of 3 patients with renal cell remaining disease free and 11 of 32 patients with melanoma remaining disease free.

Natural killer activity was not significantly affected by treatment with levamisole alone or the combination of levamisole and γ-IFN. Since levamisole is known to elevate NK activity in patients with depressed NK levels, patients were separated into two groups. Patients with low NK activity were defined as those individuals whose average NK activity from 2-3 pretreatment samples was <70 lytic units(LU) per 10^7 cells, while individuals with normal NK activity were defined as those exhibiting an average baseline NK activity of >80 LU. Baseline NK activity was comparable in the adjuvant and advanced patients populations (116 ± 11 vs. 91 ± 11), respectively. NK activity was compared on day 11 (Levamisole alone) and day 32 (11 days post initiation of Levamisole and γ-IFN). Three of seven adjuvant and two of seven advanced patients with low baseline NK activity had a rise in NK activity following Levamisole therapy. In the advanced disease patients, the rise in NK activity occurred during the combined therapy portion of the protocol and no increase was detected during Levamisole alone.

Neopterin values were elevated above baseline for all dose levels following treatment with levamisole and IFN-γ. The elevations in neopterin levels were not influenced by differing levamisole doses and were indistinguishable from those values obtained in patients treated with the same dose of IFN-γ alone on a previous protocol conducted at the BRMP. Neopterin levels increased in several patients while receiving levamisole alone. These increases occurred at all dose levels and did not appear to be dose-related. Baseline neopterin levels between groups was similar and averaged 8.8 ± 1.0 and 7.7 ± 0.9 nmol/l for patients with adjuvant and advanced disease, respectively.

SIGNIFICANCE

Levamisole in combination with 5-fluorouracil prevents the recurrence of colon cancer after surgical resection of patients with Duke's C disease. A suggestion of benefit is also observed in patients with Duke's B colon cancer. Levamisole may also benefit patients with malignant melanoma after resection, although this point is controversial. The mechanism of action of levamisole in preventing the recurrence of colon cancer remains unknown. Levamisole at doses achievable in vivo does not increase the in vitro toxicity of 5-fluorouracil for colon carcinoma cell lines. This information when combined with the known activity of levamisole in altering the immune status of patients suggests that the effect of the drug is immunologically mediated. A recent report shows that levamisole makes colon carcinoma cell lines more sensitive to lysis by NK cells.

This protocol examined the effects of levamisole administered by an alternate dose and schedule on a variety of immune functions. It is of interest that induction of neopterin was seen during the levamisole alone portion of the protocol in a subset of patients. This induction did not appear to be dose related; patients treated at all doses levels exhibited this effect. Similarly sporadic elevations of soluble IL-2 receptors were seen with levamisole alone. The significance of these findings is unclear, but it may be of interest to attempt to correlate induction of neopterin and sIL-2r in

patients with colon cancer to determine whether this correlates with continued remission. Induction of neopterin was observed reproducibly with the combination of levamisole and γ-IFN. Levamisole dose level did not affect the level of neopterin synthesis induced by γ-IFN. There was no difference in the level of neopterin synthesis observed in this trial with the combination of levamisole and IFN when compared with neopterin induced by γ-IFN alone observed in an earlier trial conducted here at the Biological Response Modifiers Program.

This trial shows that Levamisole can be given at an increased dose and a more frequent schedule than that used in most trials of this agent. Levamisole-induced bone marrow suppression is not increased by the addition of γ-IFN.

PROPOSED COURSE

This trial has completed accrual. Completion of the immunological monitoring is in progress and may allow for a more complete understanding of the immunologic effects of Levamisole.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09351-02 CRB

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.)*
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:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	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./DynCorp, Frederick, MD (W. J. Urba)

LAB/BRANCH
Clinical Research Branch

SECTION

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NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	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.)*

Approximately 20% of patients with metastatic melanoma respond to IL-2-based regimens. These responses are generally of short duration, and manipulation of the dose and schedule of IL-2 has failed to improve clinical outcome. In preclinical models, tumor-specific monoclonal antibodies (MoAb) can enhance the activity of IL-2 administered alone or in combination with lymphokine-activated killer (LAK) cells. Recently, the murine MoAb R24, which recognizes the GD3 ganglioside on melanoma tumor cells, was shown to be tolerable in phase I trials and produced some clinical responses. Therefore, we initiated a phase I trial of R24 administered by 24 hour continuous infusion on day 0 followed by IL-2 on days 1-5 (4.5 mu/m sq/d) and 11-18 (3 mu/m sq/d) and LAK cells on days 11, 12, and 14. Eight (7 evaluable) patients were treated (6 received 3 mg/m sq and 2 received 10 mg/m sq of R24). Administration of LAK cells was associated with unexpectedly severe pulmonary toxicity in 4 of these patients, suggesting that administration of the R24 prior to priming and pheresis altered the characteristics and toxicity of the LAK cells. Subsequently, we amended the protocol to delete the LAK cells and delayed the administration of IL-2 to day 14 (4.5 mu/m sq/d by IVCI for 108 hours). Twelve patients have been treated (3 at 10 mg/m sq, 6 at 30 mg/m sq, and 3 at 60 mg/m sq of R24) and dose escalation of R24 continues in order to determine a maximum tolerated dose (MTD). A partial response was seen in a patient with small skin lesions treated at the 30 mg/m sq R24 level. Tumor biopsies are obtained on all patients pre and post treatment, and preliminary observations indicate the regimen increases T-lymphocyte infiltrates in tumors.

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Principal Investigator	CTEP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	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 MTD of R24 when combined with a continuous infusion regimen of IL-2 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. Twelve hours after completing the R24 infusion on day 1, a bolus dose of IL-2 (3 mu/m^2) is given followed by a continuous infusion (4.5 mu/m^2/d) for 108 hours. On day 14-19, the course of IL-2 is repeated. Patients are evaluated for response on day 42. Biopsies are obtained pre-treatment, in between the two courses of IL-2, and 48-72 hours after the last IL-2 infusion is completed. 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 TIL. 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

During the portion of the study when LAK cells were administered, severe but reversible pulmonary toxicity was seen in 4/7 patients. In our prior experience with a similar regimen of IL-2/LAK not containing R24 and using the same dose of IL-2 during the LAK infusions, we did not observe this degree of pulmonary toxicity. The administration of R24 prior to priming and pheresis may have altered the characteristics and toxicity of the LAK cells. Flow cytometry and cytotoxicity studies on the infused LAK cells are currently in progress.

A maximum tolerated dose of R24 has not been reached in the amended study (current level 60 mg/m^2) which deleted LAK cells from the regimen and delayed the

start of the second week of IL-2 to day 14. The adverse reactions to R24 and IL-2 in this regimen are those previously characterized for each individual agent, and there is no evidence to date of additive or synergistic toxicity. A response was seen in a patient with small skin lesions at the 30 mg/m^2 R24 level, and a minor reduction in lymph node metastases was noted in a patient at the 10 mg/m^2 R24 level. Characterization of tumor biopsy specimens obtained pre and post therapy are pending, but preliminary data suggest an increase in T cell infiltrates in the tumor biopsies of most patients.

SIGNIFICANCE

This is the first trial to suggest that there may be enhanced toxicity if IL-2 and LAK therapy is given after infusion of the MoAb R24.

PROPOSED COURSE

Dose escalation of R24 will proceed until a maximum tolerated dose is reached.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09352-02 CRB

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.)*
5FU, Leucovorin, AZT & Persantine for Melanoma, Renal & Colorectal Cancer

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:	J. W. Smith II	Medical Officer	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	B. L. Gause	Medical Officer	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba)

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Clinical Research Branch

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	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 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 an AZT dose of 200 mg was chosen for the phase II study. The melanoma (14 patients) and renal cell (14 patients) portions of the phase II study have been completed and eight of a planned nine patients have been accrued to the colon cancer portion. Major toxicity was hematologic, with 13 episodes of grade III and 22 episodes of grade IV granulocytopenia. There were five episodes of greater than or equal to grade III thrombocytopenia and nine episodes of greater than or equal to grade III anemia. In renal cell carcinoma and metastatic melanoma, there have been zero complete or partial and three minor responses. In colon cancer, there has been one partial response in seven evaluable patients, with one patient too early to evaluate.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	CRB, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Barry L. Gause	Medical Officer	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 prior chemotherapy regimen, they cannot have received both 5FU and LV. 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, LV 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

The 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 an AZT dose of 200 mg was chosen for the phase II study. Six patients received AZT at 50 mg, five at 100 mg, and five at 200 mg during the phase I portion of the study. Grade IV anemia and grade IV thrombocytopenia was observed in one patient each at 50 and 100 mg dose levels, respectively. Two episodes of grade IV neutropenia and one episode of grade IV anemia were observed at 200 mg of AZT. There was one minor response at 100 mg of AZT in a patient with renal cell carcinoma.

The melanoma and renal cell portions of the phase II study have also been completed. Fourteen patients with melanoma and 14 patients with renal cell

carcinoma were entered. Major toxicity was hematologic, with 13 episodes of grade III and 20 episodes of grade IV neutropenia. There were five episodes of greater than or equal to grade III thrombocytopenia and eight episodes of greater than or equal to grade III anemia. 5FU doses were reduced in four patients were for gastrointestinal toxicity, and seven patients for cytopenias. AZT doses were reduced in three patients for gastrointestinal and 14 patients for hematologic toxicity. There were no complete or partial responses. One patient with melanoma obtained a minor response which lasted four months. Two patients with renal cell carcinoma obtained minor responses of two and six months duration.

The phase II colon cancer portion of this study is actively accruing patients. Thus far, eight patients with colon cancer have been entered onto the study. There have been five episodes of grade IV toxicity (2-neutropenia, 1-anemia, 1-mucositis), and two episodes of grade III toxicity (1-skin, 1-headache). One patient obtained a partial response and is currently receiving his 17th cycle of therapy, although he has required dose reductions of AZT and 5FU for transient anemia and hyperbilirubinemia. There were no objective responses in 6 colon cancer patients and 1 is too early to evaluate.

Laboratory studies using tritium-labelled deoxyuridine and thymidine to assess deoxyuridine and thymidine incorporation by this combination therapy regimen are pending at this time.

SIGNIFICANCE

With the addition of persantine and AZT, we had hoped to increase the objective response rate of 5FU and LV in metastatic colon cancer. Unfortunately, with seven evaluable colon cancer patients, we have not found significant antitumor activity. There is recent laboratory evidence that persantine may actually antagonize the effects of 5FU by preventing intracellular uptake of the drug. This may be a partial explanation for a disappointing activity of this regimen.

In addition, we have found this regimen to have no significant activity in renal cell carcinoma and metastatic melanoma.

PROPOSED COURSE

We have amended the protocol to stop accrual of colon cancer patients at nine if no complete responses are obtained. We are currently developing a new protocol for metastatic colon cancer combining 5FU and leucovorin with biochemical modulation by AZT and IFN-α. No further patients with metastatic melanoma or renal cell carcinoma will be treated on this protocol.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09353-02 CRB

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.)*
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 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./DynCorp, 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:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

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

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

Previous Biological Response Modifiers Program (BRMP) 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 30 mu/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.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
John W. Smith II	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 R24 given in combination with 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

In the first 21 patients treated, 19 were evaluable for response; 8 patients showed partial responses over the range of R24 doses from 5 to 100 mg/m2. This response rate appears to be much greater than our experience with the same regimen using IL-2 without R24. Major toxicity appears identical to that evidenced with the previously used regimen employing IL-2 alone, with three important additional toxicities: rapidly reversible but dose limiting laryngeal edema (2 patients), severe abdominal pain (2 patients), and moderate to severe hives (virtually all patients). Immunological studies suggest that clinical antitumor responses are accompanied with increases in serum gamma-interferon levels detectable in the 24 hours following R24/IL-2 administration. Further studies are underway to define a possible enhancement of antibody directed cellular cytotoxicity (ADCC) in responders. A protocol amendment allowing directed injection of R24 into lesions has been submitted. Production of clinical grade chimeric R24 is also underway, in the hopes of allowing longer duration of antibody treatment.

SIGNIFICANCE

The current trial suggests that R24 may enhance antitumor activity of IL-2 in melanoma. Further studies are clearly warranted to follow up on this preliminary finding.

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 09354-02 CRB

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.)*
Phase IB Evaluation of IL-1 Beta

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

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Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

See next page

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W.J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller)

LAB/BRANCH
Clinical Research Branch

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NCI-FCRDC, Frederick, MD 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.)*

Interleukin-1 beta (IL-1β) possesses antiproliferative, immunostimulatory, anti-infection, myeloprotective, and myelorestorative properties that could be beneficial in cancer treatment. In this phase I trial, IL-1β was administered IV over 15 minutes daily for seven days to patients with advanced solid malignancies. The maximum tolerated dose of IL-1β alone was 0.3 mcg/kg. A second group of patients received indomethacin plus IL-1β based on pre-clinical studies indicating that indomethacin could abrogate IL-1β-induced hypotension. The MTD of IL-1β plus indomethacin has not been determined, although one patient has had dose-limiting toxicity at 0.3 mcg/kg plus indomethacin. Fever, chills, headache, nausea, vomiting, and myalgia were commonly observed, but were not dose-limiting. Hypotension requiring IV fluids and pressors was observed at doses of 0.1 mcg/kg and above. Dose-limiting toxicities were grade IV hypotension, bronchospasm, myocardial ischemia, and atrial and ventricular arrhythmias. IL-1β treatment caused a significant dose-related increase in the total white blood count (mainly segmented neutrophils and neutrophilic bands). Bone marrow cellularity was increased after three days and seven days of treatment. Platelet counts declined during therapy, but were significantly elevated above baseline 1-2 weeks after the end of treatment. Significant increases in G-CSF and IL-6 were noted and may have been responsible for the observed hematopoietic effects. Pharmacokinetic studies indicated IL-1β to have a short half-life of 10-15 minutes. This study demonstrates that IL-1β can be safely administered to humans, that it produces biological effects very similar to those observed with IL-1α, and that important, potentially beneficial, hematopoietic effects occur at well-tolerated doses of IL-1β.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, MP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
John E. Janik	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
Barry L. Gause	Medical Officer	CRB, NCI
Robert G. Fenton	Expert	CRB, NCI
Joost J. Oppenheim	Chief	LMI, NCI
Jon T. Holmlund	Medical Officer	CRB, NCI
Brendan D. Curti	Staff Fellow	CRB, 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 doses 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

A total of 24 patients have been treated with IL-1β without indomethacin. An additional eight patients have been treated with IL-1β plus indomethacin. Toxic effects observed include nausea, vomiting, fever, chills, headache, myalgia, and arthralgia that were mostly grade II or less. Cardiovascular side effects

included hypotension that required treatment with IV fluids at doses of 0.1 mcg/kg, and both IV fluids and pressors at higher doses. Increases in liver function tests and serum creatinine were observed but were short-lived. Two patients had a grade III increase in the liver function studies and CPK. Uncommon toxicities included one patient each with hypertension accompanied by pulmonary edema, SVT, non-sustained V-tach, atrial fibrillation, and grade IV decrease in platelets. Dose-limiting toxicity was observed at 1.0 mcg/kg of IL-1β; one patient experienced grade IV hypotension and another experienced severe brochospasm and myocardial ischemia. The MTD of IL-1β alone was 0.3 mcg/kg. Patients treated with indomethacin experienced similar toxicities. One patient at the 0.3 mcg/kg dose had myocardial ischemia and pulmonary edema. A total of six patients are being entered at this dose level.

Increases in the white blood count were due to increases in the number of neutrophils and neutrophilic bands. The platelet count was increased an average of 1.6 fold above baseline 1-2 weeks after treatment. Bone marrow aspirates and biopsies obtained during and after treatment showed a significant increase in the marrow cellularity. There were no consistent changes in the frequency of CFU-GM and CFU-GEMM in the bone marrow.

Significant increases in the serum level of G-CSF were noted following the first IL-1β injection, and may have been responsible for the observed increases in the number of circulating neutrophils. IL-6 levels were also increased significantly after IL-1β treatment, and may have been responsible for the increase in the platelet count observed 1-2 weeks after the end of treatment. GM-CSF was not detected in the serum at any time point or at any dose level. Pharmacokinetic analysis revealed a short half-life for IL-1β of 10-15 minutes.

SIGNIFICANCE

This phase I study of IL-1β demonstrates that this agent, like interleukin-1 alpha (IL-1α), has a variety of important biological effects. Although IL-1α and IL-1β are distinct, proteins with only 25% homology, they do bind to the same receptor. This study indicates that IL-1β shares many of the same biological activities of IL-1α that were observed in our previous phase I trial. This protocol has demonstrated important hemopoietic effects of IL-1β including the induction of G-CSF and IL-6, as well as significant increases in bone marrow cellularity as well as increases in the peripheral neutrophil count and platelet count. Importantly, these beneficial hematopoietic effects have been noted at tolerable doses of IL-1β. Pre-clinical studies of IL-1 have indicated that this agent is one of the few agents that acts upon early stem cells in the bone marrow, and that these effects on early progenitor cells are enhanced by combinations of IL-1 with other hemopoietic factors, such as G-CSF, IL-3, and/or stem cell factor.

PROPOSED COURSE

This phase I trial is nearing completion and will define the dose range of IL-1β that is well-tolerated and produces desirable hemopoietic effects and immunologic

effects. Follow-up clinical trials of this agent are planned to determine the effects of IL-1β in conjunction with high-dose chemotherapy, G-CSF, and bone marrow transplantation in patients with relapsed Hodgkin's disease and non-Hodgkin's lymphoma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09355-02 CRB

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.)*
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:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	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.)*

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 animal models have demonstrated synergistic anti-tumor activity when agents with direct cytotoxic effects are combined with immunomodulators, 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 9 evaluable patients responded in adrenal, skin, lung, 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
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	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, 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.

Nine patients are evaluable for response. All but one patient completed at least one cycle of treatment. Three partial responses were seen in the following sites: adrenal and lymph node, lymph node and skin, and lung and lymph node. The first partial response occurred approximately 3 months after completing treatment although lesions were shrinking during two treatment cycles. The partial response has been maintained for 12+ months. The other two patients with partial responses have progressed. One patient had stable disease for 5 months after completing treatment. One other 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.

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.

SIGNIFICANCE

Although 3 partial responses were seen in the first 9 patients, we feel the regimen is excessively toxic to justify continued accrual.

PROPOSED COURSE

The protocol is closed to further accrual. Future studies will focus on combinations of chemotherapy and immunotherapy associated with less toxicity and requiring less hospitalization.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09356-02 CRB

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.)*
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:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	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./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (G. Frey)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	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.)*

Preliminary clinical trials using intravenous interleukin-2 (IL-2) at moderate or high doses concurrently with interferon-alfa (IFN) suggested that the combination could produce superior response rates in comparison to the single agents. In order to reduce acute toxicity and to test whether extending the duration of treatment would increase efficacy, we conducted a phase I study to determine the doses of IL-2/IFN that could be administered subcutaneously on an outpatient basis for prolonged periods. A secondary objective was to characterize the immunologic effects of the regimen. IL-2 was administered daily x 5 each week in all cohorts. The first cohort (IL-2 dose 3 million Roche units/m2/d) received IFN (2.5 mu/m2) TIW; only 3/6 completed at least 1 month at full doses due to chronic, disabling constitutional symptoms (fatigue, fever, nausea, and vomiting). Subsequently, all cohorts received IFN daily (in an attempt to induce tachyphylaxis to systemic symptoms), and dose escalation began at 1.5 mu/m2 of both agents. Attempts to escalate the interferon (to 3 mu/m2, 6 patients) or IL-2 (to 3 mu/m2, 4 patients) in subsequent cohorts again produced toxicity incompatible with chronic outpatient administration. A total of 16 patients were entered at the MTD (1.5 mu/m2 of both agents); 11/15 who were evaluable for toxicity tolerated outpatient treatment at full doses for at least 1 month, and 6 received from 4-9 months of treatment without dose reductions. In order to determine the role of IFN in the IL-2/IFN regimen with regard to toxicity and immune modulation, 21 patients received IL-2 alone in the first month at 1.5 mu/m2/d or 3 mu/m2/d before receiving the combination at the established MTD. Overall, 7 partial responses were seen, all in RCC patients. This regimen has substantial activity in RCC and can be administered with minimal to moderate toxicity in an outpatient setting.

PROJECT DESCRIPTION

PERSONNEL

Mario Sznol	Senior Investigator	CTEP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	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 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. To determine the contribution of interferon in the regimen, an additional two cohorts were entered who received IL-2 alone (1.5 or 3 $mu/m^2/d$) in the first cycle and IL-2/IFN at the MTD in all subsequent cycles after 2 weeks of rest. Immune parameters were drawn on the first and fifth day of treatment during weeks 1 and 2, on the first day of treatment of week 3 and the beginning of each 4 week cycle, and on the last day of treatment 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

A total of 53 patients were entered. Median age was 49 (24-78) years, male:female ratio was 29:24, Karnofsky PS was 90-100 in 85%, and histologic diagnoses were RCC (29), melanoma (12), lung (4), colon (3), and other (5).

Fifteen had either prior IL-2 or IFN treatment and a separate 14 had prior chemotherapy. An MTD was established at 1.5 mu/m^2/d of IL-2 and interferon-alfa. Of the 15 evaluable patients at this dose level, 11 were able to tolerate full doses for at least 1 month.

The regimen was active only in patients with renal cell carcinoma. Seven partial responses were seen among 29 RCC patients entered on study (28 evaluable) in lung, liver, lymph node, and retroperitoneal metastatic sites. One of these patients received only 8 doses of IL-2, and two others showed evidence of tumor regression while receiving IL-2 alone but before receiving IL-2/IFN. Four responses are ongoing, three for greater than one year.

Preliminary analyses of laboratory studies completed to date suggest that interferon decreases the peak lymphocytosis obtained with IL-2 alone, primarily by suppressing the expansion of CD56+ lymphocytes. There were no statistical differences noted in soluble IL-2 receptor, neopterin, CD3 lymphocytes, or activation of CD3 lymphocytes. None of the laboratory measures clearly correlated with response to therapy. The effects of IL-2 on circulating lymphocytes appear to be dose-related, although additional data are being gathered.

PROPOSED COURSE

After completing accrual of the cohorts receiving IL-2 alone in the first month, the study will be completed. Phase III trials in patients with RCC comparing high dose IL-2 to outpatient IL-2/IFN, and outpatient IL-2/IFN to IFN alone, are being considered in extramural centers to determine if the IL-2/IFN regimen is an effective, tolerable regimen for patients with this disease. Attempts to improve on the current level of activity of the IL-2/IFN combination are being considered. We are planning to combine IL-2/IFN with adoptively transferred OKT3 activated cells, or with other biologic agents (such as vaccines or the cytokine IL-6) capable of inducing specific cell-mediated immunity to RCC antigens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09357-02 CRB

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.)*
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	BRMP, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI
	R. G. Fenton	Expert	BRMP, NCI
	W. H. Sharfman	Expert	BRMP, NCI
	K. C. Conlon	Senior Staff Fellow	BRMP, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Treatment 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:
3.5	2.0	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.)*

Interferon induces the synthesis of a large number of gene products. These products play a role in the antiviral and antiproliferative effects of alpha interferon. Two of the induced gene products, 2',5'-oligoadenylate synthetase and the double-stranded inhibitor (DSI) are induced by interferon but require double-stranded RNA to activate them. The DSI plays a major role in inhibiting protein synthesis initiation by phosphorylating the eukaryotic initiation factor 2 (eIF-2) on serine 51 of its alpha subunit.

This study combined administration of interferon alpha and Poly ICLC, a form of double-stranded RNA in order to determine the optimal dose level of interferon for induction of the DSI and the optimal dose of Poly ICLC for activation of the kinase. Three dose levels of interferon were combined with one of four dose levels of Poly ICLC.

This study was designed to determine the toxicity of this combination and the maximal tolerated dose. Dose limiting toxicity was observed with the combination of interferon 10 mu/m^2 and Poly ICLC 0.03 mg/m^2. Toxicity consisted of prolonged hypotension requiring hospitalization for intravenous fluid administration. Higher doses of Poly ICLC allowed administraion of interferon at the 10 mu/m^2 dose level without this toxicity. These results suggest that Poly ICLC may be more toxic at lower doses or that higher doses of ICLC may reduce the toxicity of interferon.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

PROJECT DESCRIPTION

PERSONNEL

Ronald. G. Steis	Medical Officer	BRMP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	BRMP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Robert G. Fenton	Expert	BRMP, NCI
William H. Sharfman	Expert	BRMP, NCI
Kevin C. Conlon	Senior Staff Fellow	BRMP, 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 the antitumor effects of Poly-ICLC and interferon alpha used in combination.

5. To determine in a preliminary fashion the effect of Poly ICLC on the plasma pharmacokinetics of interferon.

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

Sixty-three patients have been treated with interferon alpha and Poly ICLC. Two histologies, renal cell carcinoma and malignant melanoma comprise the majority of patients treated on this study. The median age of the patients was 53 with a range of 25 to 77 years. Twenty-eight women and 35 men were treated.

The toxicity profile of the combination was not significantly altered by the addition of Poly ICLC at the higher doses of Poly ICLC used in this trial (0.1-1 mg/m^2). The expected toxicities of interferon alpha, fever, chills, myalgias, arthralgias, fatigue, anorexia, headache, nausea, vomiting, granulocytopenia, liver function tests abnormalities and thrombocytopenia were observed. The toxicity was dose related and increased in frequency and severity with increasing doses of interferon. Three patients were removed from study during the interferon alpha alone portion of the protocol. One patient at the 3 million u/m^2 interferon dose level experienced grade IV liver function test abnormality and two patients treated at the 10 million u/m^2 interferon alpha dose level were removed from study; one for severe fatigue and the second for protracted orthostatic hypotension requiring hospitalization and i.v. fluid administration.

Dose-limiting toxicity was observed in three of four patients treated with the combination of interferon at a dose of 10 million u/m^2 and Poly ICLC at a dose of 0.03 mg/m^2. Toxicity consisted primarily of fatigue and orthostatic hypotension requiring intravenous fluid administration. Hypotension occurred in three of four patients treated at this dose level. This was the lowest dose of Poly ICLC used and was tested after completing the higher doses of Poly ICLC, 0.1 to 1 mg/m^2, employed in this trial. Two possible explanations arise to explain the apparent increased toxicity of low doses of Poly ICLC. First, Poly ICLC may have increased toxicity at the lower dose levels. Alternatively, Poly ICLC may ameliorate interferon induced toxicities and allow administration of increased doses of interferon.

Fifty-two patients are evaluable for tumor response. The remaining eleven patients are either too early in the course of treatment to assess response, or were removed from study during the interferon alpha alone portion of the study for toxicity or were removed from study for non-compliance. Three partial responses have been observed with the combination of Poly ICLC and interferon. One patient each with renal cell cancer, rectal cancer, and malignant melanoma achieved a partial response. Two additional patients, both with renal cell cancer, achieved minor responses.

The patient with rectal cancer had nodal metastases in the anterior cervical and supraclavicular region, abdominal nodal metastases and hepatic metastases. This patient achieved a partial remission after four months of therapy and maintained that response for six months, but then had progressive disease with new nodal metastases. The patient with renal cancer had a renal bed mass and pericolonic metastases and achieved a partial remission after two months of combined therapy and maintains this response at four plus months of therapy. The patient with malignant melanoma had skin and breast metastases and achieved a partial remission after four months of therapy that has lasted for 12+ months. Two additional patients, both with renal cell cancer, achieved minor responses and

maintained their responses for 12+ and 6+ months. All of the responding patients have been treated with relatively low doses of interferon (1 or 3 million $u/m^2/d$) except for the patient with rectal carcinoma, who was treated with 10 million $u/m^2/d$ of interferon alpha.

SIGNIFICANCE

An intriguing finding from this trial is the recent observation that patients treated with low doses of Poly ICLC have an apparent increased toxicity in comparison with the higher doses employed in this trial. If these higher doses of Poly ICLC (0.1-1 mg/m^2) can protect against the long-term toxicity of interferon, it may be possible to increase the dose of interferon that patients are able to tolerate on a daily basis, thereby allowing for a more effective delivery of interferon to patients.

PROPOSED PLANS

Although the response rate observed on this trial is relatively low, approximately 6% in this group of patients with metastatic disease of various cancers, we would like to pursue the idea of inhibiting protein synthesis initiation and attempting to correlate drug sensitivity with levels of eukaryotatic initiation factor activity levels. It is known that the guanine nucleotide exchange factor or eIF-2B is a limiting factor in initiation of protein synthesis in eukaryotatic cells. Cells with high levels of eIF-2B activity easily overcome the block to protein synthesis initiation produced by phosphorylation of eIF-2α. The activity of eIF-2B can be modulated by the level of polyamines present in cells. Difluoromethylornithine, an ornithine decarboxylase inhibitor, alters the synthesis of putrescine and spermidine, polyamines that are essential to the function of eIF-2B. Difluoromethylornithine may augment the antitumor effect of the combination of interferon alpha and Poly ICLC by altering the level of eIF-2B in cells. A significant problem associated with DFMO inhibitors is that polyamines present in the diet can have significant effects on the levels of polyamines present in the blood. Polyamine analogues are being synthesized at the present time and could be employed in future trials as more specific inhibitors of the activity of eIF-2B.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09361-01 CRB

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.)*: Carboplatin, Cisplatin, IFNα and Tamoxifen for Metastatic Melanoma

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

PI:	W. H. Sharfman	Expert	CRB, NCI
Others:	J. W. Smith II	Medical Officer	CRB, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. E. Janik	Expert	CRB, NCI

COOPERATING UNITS *(if any)*: Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); 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:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

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

Recent reports indicate that combination chemotherapy may have more activity than single agent DTIC in metastatic melanoma. In particular, the regimen of DTIC, cisplatin, BCNU and tamoxifen, as well as the regimen of interferon-alpha and cisplatin have both shown promising activity. In this trial, we have combined what may be the essential elements of each of these two regimens into an outpatient regimen of cisplatin, interferon-alpha and tamoxifen. Carboplatin has been added to provide higher total doses of platinum.

The drugs were administered in a 28 day cycle as follows: carboplatin, 400 mg/m2 IV day 0, cisplatin, 25 mg/m2 IV day 7, 14, 21, tamoxifen, 20 mg po bid, and interferon-alpha 5 million units/m2 TIW. Eight patients are currently evaluable for toxicity and response. Thirteen cycles of therapy have been administered. The major toxicity was hematologic with four episodes of ≥ grade III neutropenia, four episodes of ≥ grade III thrombocytopenia, and one episode of grade III anemia. Five patients required treatment delays or dose adjustments of platinum or interferon-alpha for hematologic toxicity. Two patients had greater than or equal to grade II nausea and vomiting. Four patients complained of worsening fatigue with therapy. There has been one partial response, two mixed responses, and five patients demonstrated progressive disease. With one patient yet to be evaluated, it appears that this regimen does not have significant activity in melanoma.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

William H. Sharfman	Expert	CRB, NCI
John W. Smith II	Medical Officer	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John E. Janik	Expert	CRB, NCI

OBJECTIVES

1. To determine the toxicity of an outpatient regimen of cisplatin, carboplatin, tamoxifen and interferon-alpha.

2. To determine the antitumor activity of an outpatient regimen of cisplatin, carboplatin, tamoxifen and interferon-alpha.

METHODS EMPLOYED

Eligible patients must have a histologically documented diagnosis of metastatic melanoma and must not have received platinum-containing chemotherapy regimens. Patients were evaluated at the Clinical Research Branch, Biological Response Modifiers Program, in Frederick, Maryland. Treatment was administered in the outpatient clinic and consisted of 28 day cycles of the following: carboplatin 400 mg/m^2 IV day 0, cisplatin 25 mg/m^2 IV day 7, 14, 21, tamoxifen 20 mg po bid, and interferon-alpha 5 million units/m^2 TIW. Doses of cisplatin were held for platelet counts less than 50,000 or total white counts less than 1,000. Patients were evaluated after two cycles of therapy and continued on treatment only if there was evidence of a response.

MAJOR FINDINGS

Ten patients have been entered on this study. Eight patients are currently evaluable, one patient took herself off the study after one week, and one patient is too early to evaluate. Thirteen cycles of therapy have been administered. The major toxicity was hematologic with four episodes of $\geq$ grade III neutropenia, four episodes $\geq$ grade III thrombocytopenia, and one episode of grade III anemia. Hematologic toxicity caused treatment delays or dosage reductions of platinum or interferon-alpha in five patients. One patient required a decrease in carboplatin due to a decrease in creatinine clearance below 60 milliliters per minute. The same patient also required a decrease in interferon-alpha dose because of depression. Four patients complained of worsening fatigue with therapy. Two patients had greater than or equal to grade II nausea and vomiting. Three patients received only one cycle of therapy, one because of progressive disease and two because of a combination of nausea, vomiting, and fatigue. Thus far, one patient has obtained a partial response, two patients have had mixed responses with decreasing lung lesions but enlarging subcutaneous lesions, and five patients have developed progressive disease.

SIGNIFICANCE

With one patient still to be evaluated, it does not appear that this regimen will have significant activity against metastatic melanoma. It should be noted that many of the patients began the study with a decreased performance status and an extensive tumor burden which may partially explain the low level of activity of the regimen. In addition, recent reports indicate that there may be a potential antagonism between tamoxifen and interferon-alpha.

PROPOSED COURSE

We plan to amend this protocol to either add DTIC or higher doses of tamoxifen to the basic platinum and interferon regimen. Carboplatin may have to be replaced by higher doses of cisplatin to avoid more hematologic toxicity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09362-01 CRB

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.)*
Phase I Evaluation of Intratumoral Interleukin-1 Alpha

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

PI:	J. E. Janik	Expert	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W.J. Urba)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

The purpose of this protocol is to determine the maximum tolerated dose (MTD) of intralesional interleukin-1 (IL-1) alpha. We plan to examine tumor specimens obtained before and after intralesional injection of IL-1 using immunohistochemical techniques. The tumor response in the injected lesion and in distant metastasis will be monitored.

A dose escalation scheme with half-log increments will be used to determine the MTD. The starting dose used was 0.001 μg/kg and patients are currently being treated at the 0.1 μg/kg dose level. The MTD of IL-1 alpha has not been determined to date.

Patients treated with intralesional IL-1 alpha experienced similar toxicities to those observed with intravenous IL-1 alpha. Biopsy specimens of lesions injected with IL-1 show evidence of vasculitis with lymphoid aggregates surrounding vessels outside the injected tumor nodule and occasionally vessels within the tumor itself. Tumor biopsies have shown evidence of intratumoral lymphoid aggregates that were not identified on pretherapy biopsy specimens. Neither local nor systemic antitumor responses have occurred in this initial group of 15 patients.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

PROJECT DESCRIPTION

PERSONNEL

John E. Janik	Expert	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	CRB, NCI
William H. Sharfman	Expert	CRB, NCI

OBJECTIVES

1. To determine the toxicity and MTD of IL-1 alpha given by intratumoral injection.

2. To examine injected tumor specimens for evidence of inflammatory response.

3. To determine whether IL-1 induces any serologic antitumor response following intratumoral injection.

4. To note any systemic antitumor response associated with local therapy.

METHODS EMPLOYED

Patients were eligible for this trial if they had a histologically confirmed solid tumor which was not curable by standard treatment regimens. All patients were required to have at least two sites of tumor in an easily accessible site amenable to intralesional injection. The first site was used as a pretherapy biopsy specimen and the second lesion was available for intralesional therapy. Patients were required to be greater than 18 years of age and 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 arrhythmias requiring treatment, no history of central nervous system metastasis, no history of rheumatoid arthritis, systemic lupus erythematosus, inflammatory polyarthritis, adrenal insufficiency, or other autoimmune disorders. Patients must have been without any other cancer treatment for four weeks prior to entry on this study. The initial clinical evaluation included a complete history and physical examination, a serum chemistry profile, complete blood count with differential and platelet count, coagulation studies, a urinalysis, and EKG with cardiac stress tests, pulmonary function tests including ABG and DLCO, chest x-ray, CT of the brain, baseline immune parameters, serum iron, total iron binding capacity, ferritin and erythropoietin. Imaging studies, performed as clinically indicated, were obtained prior to therapy for tumor assessment. Patients received therapy according to the following plan: All patients received IL-1 alpha intralesionally daily for five days. After a three day or three week rest period off therapy, the injected lesion was biopsied. Patients who showed evidence of a response within the IL-1 injected lesion were eligible for further intralesional IL-1 alpha therapy. Patients who had their lesion biopsied at three days following completion of therapy were eligible for additional therapy if they had any other lesions that could easily be injected. Patients were treated in cohorts of three at escalating doses starting at a dose

of 0.001 μg/kg and the dose escalated with half-log increments. Patients are currently being treated on dose level 5 with a dose of 0.1 μg/kg. In addition to serial complete blood counts and serum chemistry studies, patients will have serial iron studies performed to determine the effect of IL-1 alpha on the synthesis of ferritin and transferrin. Tumor biopsy specimens will be examined for the expression of tumor antigens, class I and class II major histocompatibility complex antigens, infiltration of effector cells and their phenotypic characterization using immunoperoxidase staining. Serum will be examined for the development of antibodies specific to the patients tumor using western blotting techniques.

MAJOR FINDINGS

This protocol enrolled 15 patients. All patients are eligible for toxicity assessment and disease response. There were 9 men and 6 women treated. The median age of the patients was 57 with a range of 34 - 72 years. Twelve patients had malignant melanoma, two colorectal carcinoma, and one renal cell cancer. The toxicity profile of IL-1 alpha administered by intralesional injection is comparable to that observed with intravenous administration. Toxicity has been relatively minor with this method of administration. Fever and chills were common, but grade II or less in all but one patient who had grade III fever. Two of the patients treated at the lowest dose level 0.001 μg/kg did not experience fever. Nausea, vomiting, headache, myalgia, arthralgia, and abdominal pain were less commonly observed and were grade I in all cases except for a single patient with grade II nausea.

There was evidence of erythema at the site of IL-1 and injection in 8 of 15 patients. In 7 of these patients, the erythema was transient and lasted only one or two days. A single patient developed diffuse erythema of the right leg after IL-1 injection at the 0.1 μg/kg dose level. This appeared clinically to be a cellulitis and IL-1 therapy was discontinued after the first dose; he was treated with dicloxacillin with a gradual resolution of his fever and erythema. Therapy was resumed one week after the initial injection and the patient had a prompt recurrence of right leg erythema. IL-1 therapy was continued for 2 additional doses but was discontinued because of skin breakdown in the skin overlying the injected lesion. The patient had persistent fever and erythema of the right leg after stopping therapy. There was no evidence of systemic bacterial infection and with an alteration in his antibiotic coverage there was a gradual resolution of his fever and skin erythema. A biopsy of the erythematous skin in the right leg showed evidence of vasculitis with lymphoid infiltrates. An additional patient treated at the 0.03 μg/kg dose level developed a Clostridium septicum cellulitis requiring surgical debridement and intravenous antibiotics. It was felt in retrospect that the cause of the Clostridium septicum cellulitis may have been due to gastrointestinal tract involvement by melanoma as the patient had a diagnosis of small bowel metastases six months after completion of IL-1 alpha therapy.

No alterations in the blood counts have been observed at the 0.001 - 0.03 μg/kg dose levels. At the 0.1 μg/kg dose, increases in white blood count were observed in all patients without change in platelet counts. No renal or hepatic toxicity was observed. There have been no minor or partial responses with doses ranging from 0.001 - 0.1 μg/kg. Five of 15 patients had stable disease in the

injected lesion. Five patients had progressive disease and in five other cases, the patients lesions were biopsied thereby precluding evaluation of the injected lesion. In all five of these later cases, the patients had evidence of progressive disease in other lesions. A single patient treated at the 0.01 μg/kg dose level had evidence of progressive disease in the IL-1 injected lesion but had a 70% reduction in the size of two other subcutaneous nodules. Because of the evidence of response in these lesions, he received an additional cycle of intralesional therapy in the responding lesion. There was evidence of progressive disease in this initially responsive lesion following direct intralesional injection.

The biopsy specimens obtained from patients at the higher dose levels showed evidence of vasculitis in vessels surrounding the injected tumor nodules. Vasculitis in tumor vessels has occasionally been observed. This change appears to be related to IL-1 alpha administration since patients treated at the lower dose levels did not exhibit these changes. Occasional patients have shown lymphoid aggregates in the post-therapy biopsy specimens that were not identified pretherapy, suggesting that IL-1 can induce some proliferation of lymphocytes at the site of the injected lesion. There was no infiltration of the injected lesion by polymorphonuclear leukocytes or monocytes. Class I expression was positive before therapy in all lesions and was not obviously affected by IL-1α. HLA-DR expression was positive in some lesions before therapy and was unaffected by IL-1 injection. No lesion became positive for HLA-DR expression following IL-1α therapy.

SIGNIFICANCE

In contrast to the results obtained with other biological response modifiers, IL-1 alpha administered intralesionally does not exhibit significant antitumor activity at the doses tested. Response rates of 50 - 70% have been reported after administration of BCG, interferons alpha, beta and gamma. It is possible that higher doses of IL-1 alpha will show activity when administered locally. Local production of IL-1α may play a significant role in the development of systemic vasculitis. Clinical trials of IL-1 receptor antagonists may be warranted in vasculitis based on the ability of IL-1α to induce lymphoid vasculitis.

PROPOSED PLAN

We will continue to accrue patients on this study to determine the MTD of IL-1 alpha administered intralesionally. After the MTD has been determined, attempts to augment this therapy by two means will be employed. First, the lesion to be injected with IL-1 will be irradiated to induce cell death. It is possible that IL-1 can act more effectively as an adjuvant in this setting. Second, patients will be treated with IL-2 and alpha-interferon by subcutaneous injection in an attempt to induce proliferation of T cells that may have been stimulated by the IL-1 alpha injections. The appropriate time to obtain biopsy specimens following IL-1α injection is unclear. There are suggestions that the effects of IL-1 injection are reduced with repetitive doses. Biopsies will be obtained in selected patients after the 1st dose and at various times during the 1st week of therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09363-01 CRB

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.)*
Measurement of Interstitial Pressure in Subcutaneous Tumor Sites

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

PI:	B. D. Curti	Senior Staff Fellow	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. W. Smith II	Medical Officer	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	J. E. Janik	Expert	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Frederick Memorial Hospital, Frederick, MD (P. Kremers)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Interstitial pressure is a physiologic property of the fluids which occupy the interstitial space of normal and tumor tissues. Animal models have suggested that these pressures are extremely high in tumors and may present a significant barrier to the movement of chemotherapy and immunotherapy agents into a tumor nodule.

This goal of this study is to measure the interstitial pressure of tumors in patients who are enrolled in immuno- or chemotherapy trials at the BRMP. Additional data on the histologic diagnosis, the size of the nodule and its response to treatment will be examined in relation to the interstitial pressure. Six patients have been evaluated thus far, pressures as high as 60 mmHg have been observed. The only responding lesion in which pressure was measured, was a melanoma with a pressure of 10 mmHg; this value was 5-fold lower than other non-responding melanoma nodules. No toxicities directly attributable to the measurement procedure have been seen.

PROJECT DESCRIPTION

PERSONNEL

Brendan D. Curti	Senior Staff Fellow	CRB, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John W. Smith II	Medical Officer	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
John E. Janik	Expert	CRB, NCI

OBJECTIVES

1. Determine the interstitial pressure of subcutaneous and lymph node metastases of various tumor histologies.

2. Determine if correlations exist between interstitial pressure and tumor size, histology and response to treatment.

3. Determine if changes in interstitial pressure occur during chemo- or immunotherapy.

METHODS EMPLOYED

Any patient with a histologically confirmed diagnosis of metastatic malignancy and who can participate in any other BRMP treatment protocol is eligible. In addition to the laboratory tests and radiologic studies required by their primary treatment protocol; the coagulation status of the patient is assessed with a prothrombin time, partial thromboplastin time and a template bleeding time. Ultrasound examination of the subcutaneous nodules or palpable lymph nodes of interest are obtained before and after interstitial pressure measurements. Interstitial pressure is measured by a wick-in-needle technique. The measurement needle employed is connected to a pressure transducer and chart recorder which are calibrated in mmHg. To obtain a measurement, the needle is placed directly into the tumor. After an equilibrium pressure is recorded, the needle is withdrawn in 1 mm increments with additional readings noted. Some patients have the measurement needle left in place while they are receiving their protocol immunotherapy or chemotherapy agents. These procedures are repeated with each subsequent treatment cycle and when the patient goes off study.

MAJOR FINDINGS

To date, 6 patients have been enrolled on the study; five with melanoma and one with indolent lymphoma. Interstitial pressures between 40-60 mmHg were found in 4 of the melanoma patients. None of these lesions have responded to a variety of immunotherapy agents. One melanoma lesion had a pressure of approximately 10 mmHg. This lesion had a greater than 50% reduction in size with IL2 and R24 monoclonal antibody treatment. The lymphoma also had a pressure of 10 mmHg. This patient is on a watch and wait arm of BRMP protocol 8903 and has not yet received any chemotherapy or radiation.

There have been no toxicities observed thus far that can be directly attributed to this protocol. One patient had the appearance of a secondary nodule on a lesion in which interstitial pressure was measured. This was felt to be secondary to progressive disease and not a sequela of the measurement needle.

SIGNIFICANCE

Interstitial pressure can be safely and routinely measured in subcutaneous and lymph node sites. In some malignant lesions, very high pressures have been seen. It appears that tumor nodules with low pressures may be more responsive to chemotherapy or immunotherapy regimens however, more data are needed to substantiate this finding.

PROPOSED COURSE

The study remains open with a goal of accruing 40 patients. Further data will be gathered not only on static interstitial pressures but also on dynamic measurements obtained while the patient is receiving their chemotherapy or immunotherapy agent.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09364-01 CRB

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.)*
Role of IL-2 in Active Specific Immunotherapy for Renal Cell Carcinoma

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

PI:	R. G. Fenton	Expert	BRMP, NCI
Others:	J. W. Smith II	Medical Officer	BRMP, NCI
	W. H. Sharfman	Expert	BRMP, NCI
	D. L. Longo	Associate Director	OAD, BRMP, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba)

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.)*

This protocol was designed to test whether IL-2 can act as an immunologic adjuvant in the active specific immunization of patients with metastatic renal cell cancer.

Stage IV patients whose primary renal tumors have not been removed or who have otherwise easily accessible tumor are taken to the operating room, where tumor is obtained and processed into a single cell vaccine. After recovering from surgery, patients are given 3 autologous tumor vaccines at weekly intervals. The first two vaccines consist of BCG and autologous tumor cells and the final vaccine is tumor cells alone. Patients are randomized to receive no IL-2, low-dose IL-2 or high-dose IL-2 administered subcutaneously during the vaccination period.

Twelve patients have been treated on this protocol, of whom seven are evaluable for delayed type hypersensitivity (DTH) testing. Two of seven developed positive DTH responses. Despite this, no patient had disease regression. However, one patient who was subsequently treated with subcutaneous IL-2 had a dramatic response, perhaps reflecting potentiation of a subclinical vaccine effect.

PROJECT DESCRIPTION

PERSONNEL

Robert G. Fenton	Expert	BRMP, NCI
John W. Smith II	Medical Officer	BRMP, NCI
William H. Sharfman	Expert	BRMP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI

OBJECTIVES

1. To determine whether IL-2 given as an adjuvant significantly potentiates the immunologic response of patients with renal cell carcinoma to an autologous tumor cell vaccine.

2. To determine the toxicities of such a regimen.

3. To record any antitumor responses.

METHODS EMPLOYED

Surgery

Patients with metastatic disease undergo simple or radical nephrectomy (to obtain sufficient tissue for the vaccine and obtain local control if possible, but not done with curative intent) at Frederick Memorial Hospital. The bulk of tumor tissue is preserved and transported to Biotechnology Research Institute (Rockville, Maryland), where the tumor is processed, and single cell suspensions made from the tumor as well as normal adjacent renal parenchyma. These are frozen and utilized as irradiated vaccine and controls, respectively.

Vaccinations

Two to four weeks postoperatively, patients are randomized to one of three treatment groups receiving either no IL-2, low dose IL-2, or high dose IL-2 subcutaneously for five days following each of 3 weekly autologous tumor vaccinations. Arm A receives no IL-2, Arm B receives IL-2 (human recombinant IL-2 from Cetus Corporation) at doses of 1.2×10^6 IU/m^2/d; and Arm C receives IL-2 at 1.2×10^7 IU/m^2/d. (Note: in "Cetus units" Arm B equals 2×10^5 u/m^2/d, and Arm C equals 2×10^6 u/m^2/d). All IL-2 doses are given subcutaneously. Intradermal vaccines consist of a suspension of 10^7 lethally irradiated autologous tumor cells plus 10^7 BCG mixed on the day of the vaccination (Day 0). The first inoculation is given intradermally in the right anterior thigh approximately 10 cm below the inguinal lymph nodes, and the second is given on Day 7 in the corresponding location in the opposite thigh. The third (and final) vaccination is given on Day 14 intradermally over the left deltoid muscle and contains 10^7 autologous tumor cells but no BCG. Patients randomized to receive IL-2 receive the initial IL-2 dose (Day 0) two hours after the intradermal administration of the vaccine; this is given subcutaneously immediately under the vaccination site (which is intradermal). Subsequent IL-2 injections (Day 1-4

each week) are given in the same extremity that received the vaccination. Injections are given q24 hours on Day 0-4, Day 7-11, and Day 14-18.

Beginning on Day 28, patients are tested for DTH responses by intradermal injections of 10^4, 10^5, and 10^6 irradiated autologous tumor cells and 10^4, 10^5, and 10^6 normal autologous renal cells at separate sites on the volar surfaces of their forearms. Responses are evaluated 24 and 48 hours later. A positive DTH test is defined as induration (not erythema) measuring $\geq$ 25 mm^2 overall (determined by multiplying biperpendicular diameters), as measured at 24 or 48 hours, or both. Patients have the initial post-vaccination DTH test on Day 28; and are DTH tested on each follow-up visit to the BRMP. All patients have their tumor reevaluation eight weeks after the initial vaccination (Day 56), and each month thereafter.

MAJOR FINDINGS

Twelve patients have been enrolled on 8919 since its approval in September 1990. One patient died of rapidly progressive RCC prior to randomization or vaccination; two patients had autologous vaccines produced from metastatic deposits and hence had no normal renal tissue for controls in DTH tests (1 has progressive disease, 1 is stable); and two other patients are too early to evaluate. Of the remaining 7 patients, three were randomized to receive no IL-2, and two each to low- and high-dose IL-2. Three patients have died of progressive disease, three patients with progressive disease after completing the protocol are receiving other therapies, and one patient is currently being followed post-vaccination. Two of the seven (29%) vaccinated patients developed positive DTH responses to autologous tumor when compared to normal renal tissue controls, one from the no IL-2 arm and one from the high-dose arm. In both cases, DTH tests were negative at day 28 (ten days after the last vaccination) but were positive when tested at day 56. This could be due to the masking of antitumor immunity by an early and transient immune response against DNAse and/or collagenase (which contaminate both the normal kidney and tumor single cell suspensions); this reactivity may wane by day 56 unmasking the more long-lived tumor-antigen specific responses. Alternatively, maximal antitumor reactivity may occur after the day 28 test. Of note, the surviving DTH(+) patient was DTH(-) when retested 5 months after the positive day 56 test.

Toxicities from vaccination included induration and ulceration at the vaccine sites when BCG was included in the vaccine preparations (Day 0 and Day 7). The ulcers were self limited and required only local therapy. Regional lymph nodes draining vaccination sites were generally not enlarged or tender; this is in marked contrast to the responses seen in the colon autologous tumor vaccination studies with patients in the adjuvant setting. IL-2 therapy associated with the vaccine was well tolerated. No systemic BCG infections have been observed.

It is our intention to treat patients who have progressive disease following the vaccination protocol with an IL-2-containing regimen with the hope of expanding tumor-specific lymphocytes generated by the vaccine. Four patients with PD have been enrolled on BRMP protocol 8905, receiving daily subcutaneous injections of IL-2 (at 1.5 or 3.0 million units/m^2). Two patients continue on this therapy and have stable disease, and 1 patient has progressed. The other patient has had a remarkable clinical course. This 50 year old man had residual multiple pulmonary

metastases (largest 2 cm) following right nephrectomy for vaccine production. His KPS was 100%. He received three vaccinations without IL-2, and became strongly DTH positive on day 56 (at challenge doses of 10^6, 10^5 and nearly positive at 10^4).

However at the day 56 evaluation, he had progressive disease (67% increase in the sum of the products of indicator lung metastases). He was begun on BRMP protocol 8905 and received eight doses of subcutaneous IL-2 (3 mu/m^2) at which time he developed severe, diffuse muscle pain. His CPK rose to 20,000, and a muscle biopsy demonstrated a necrotizing myopathy suggestive of an autoimmune process. This is the first case of necrotizing, myositis seen in any IL-2-treated patient at the BRMP. The IL-2 was stopped, and the clinical syndrome rapidly resolved and has not recurred. Chest x-ray at that time showed stable disease with many asymptomatic 2-3 cm pulmonary lesions. During the next three months the patients' pulmonary lesions decreased by 87% (off therapy). Currently, he has two stable lesions of 1-1.5 cm and other faint residual densities. We continue to follow him bi-monthly. Repeat DTH tests done five months after the Day 56. Tests were negative at all doses of tumor cell challenge.

SIGNIFICANCE

It has been possible to generate autologous tumor vaccines from all patients enrolled on the protocol. Therapy has been well tolerated, with the vaccinations and IL-2 treatment being performed on an outpatient basis. It appears that it is possible to generate reactivity against autologous tumor in a minority of patients with advanced renal cell cancer.

PROPOSED COURSE

The initial impression is that IL-2 does not potentiate DTH responses to autologous tumor in patients with advanced renal cell cancer. Of the initial seven patients treated, four had bone metastasis prior to therapy and underwent rapid clinical deterioration. Therefore, we intend to treat three or four patients with relatively low bulk disease (i.e. primary tumor plus lung metastases) to better determine if IL-2 can act as an adjuvant. In a similarly designed protocol, IL-1 will be tested for adjuvant effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09365-01 CRB

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.)*
Phase I Study of High Dose Carboplatin Plus IL-1α

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

PI:	J. W. Smith II	Medical Officer	BRMP, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	J. E. Janik	Expert	BRMP, NCI
	W. H. Sharfman	Expert	BRMP, NCI

See next page

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program NCI, Bethesda, MD (M. Sznol, L. Miller); Data Management Services, NCI-FCRDC, Frederick, MD (W. G. Alvord)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Thrombocytopenia induced by high-dose chemotherapy remains a significant clinical problem that limits dose intensification of many chemotherapy agents. In our phase I trial of IL-1α, we noted increased megakaryocytes in the bone marrow and an increase in the platelet count after IL-1α treatment. We designed the present trial to determine if IL-1α could ameliorate the thrombocytopenia induced by chemotherapy. We chose carboplatin to combine with IL-1α because it has considerable antitumor activity, but is limited by bone marrow suppression, especially thrombocytopenia. This study has enrolled 27 patients to date. Seven patients were in a control group and received carboplatin alone (800 mg/m^2). Ten patients received IL-1α at 0.03 mcg/kg and ten received 0.1 mcg/kg IL-1α with half the patients receiving IL-1α before and half after carboplatin (800 mg/m^2). Toxicities observed in this trial were similar to those observed with previous trials of either agent alone. IL-1α treatment appears to have lessened the degree of thrombocytopenia in some patients, but this effect has not been consistently observed at the first two dose levels of IL-1α. Enrollment of 10 patients at the highest IL-1α dose (0.3 μg/kg) will be completed in the near future. If IL-1α treatment lessens the bone marrow suppression of carboplatin, a second part of this study will determine to what extent IL-1α can increase the dose intensity of carboplatin.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Medical Officer	BRMP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
John E. Janik	Expert	BRMP, NCI
William H. Sharfman	Expert	BRMP, NCI
Barry L. Gause	Medical Officer	BRMP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Jon T. Holmlund	Medical Officer	BRMP, NCI
Brendan D. Curti	Staff Fellow	BRMP, NCI
Robert G. Fenton	Expert	BRMP, NCI

OBJECTIVES

1. To determine the toxicity of the combination of IL-1α and high dose carboplatin.

2. To determine whether IL-1α can limit the bone marrow suppression caused by high dose carboplatin.

3. To determine the best sequence for giving IL-1 in relationship to high dose carboplatin, that is, whether giving IL-1α before high dose carboplatin or giving IL-1α after high dose carboplatin results in better effects on peripheral granulocyte and platelet counts after treatment with carboplatin.

4. To determine the dose of IL-1α that is optimal for limiting bone marrow suppression by high dose carboplatin.

METHODS EMPLOYED

Eligibility criteria for this trial included histologically confirmed tumor that was not curable by surgery, radiotherapy, or standard chemotherapy. Patients had to have failed standard treatment regimens or have a malignancy for which no effective treatment existed. Patients were required to have a Karnofsky performance status $\geq$ 70%, life expectancy of at least 3 months, evaluable or measurable disease, and adequate physiological function. Prior radiation therapy or chemotherapy with mitomycin-C or nitrosoureas were exclusion criteria.

The first cohort of patients treated in this study was given carboplatin alone at 800 mg/m^2 without any IL-1. These patients served as a control group. Subsequent patients were randomly assigned to treatment with IL-1α before carboplatin or IL-1α after carboplatin. Three dose levels of IL-1 in each group were studied, 0.03 mcg/kg, 0.1 mcg/kg and 0.3 mcg/kg. IL-1α was given by daily IV infusion over 15 minutes for 5 consecutive days beginning one day after carboplatin or for 5 days before carboplatin ending two days prior to the administration of carboplatin. At least five patients were enrolled in each before and after group before dose escalation of IL-1 was continued.

Treatment was given every 28-35 days and tumor response was evaluated every two months. Stable or responding patients could continue treatment until they had evidence of progressive disease. Patients whose platelet count dropped below 20,000/μL received platelet transfusions. After treatment at all dose levels and schedules is complete, a statistical analysis will be performed to determine which dose and schedule (if any) of IL-1 significantly reduced the severity and duration of granulocytopenia and thrombocytopenia. If an optimal dose and schedule of IL-1 can limit the bone marrow suppression due to carboplatin, then a second part of this protocol will begin where carboplatin is escalated in subsequent cohorts of patients to 1,200, 1,600, and 2,000 mg/m^2 until hematologic toxicity becomes excessive.

To date, seven patients have been entered into the control group, five patients each into the 0.03 mcg/kg IL-1α before and after groups and five patients each into the 0.1 mcg/kg IL-1α before and after groups.

MAJOR FINDINGS

Carboplatin treatment has been well tolerated without severe nausea or vomiting, and no neurotoxicity or nephrotoxicity. One patient who had received prior cisplatin had grade II hearing loss. Carboplatin treatment was given in the outpatient setting and no patient required hospitalization because of side effects due to carboplatin therapy. IL-1α treatment produced toxicities similar to that observed in our previous phase I trial and our phase II trial. One patient experienced myocardial ischemia with his first dose of IL-1α and received no further treatment with IL-1. Another patient developed a decline in his ejection fraction and congestive heart failure with his first dose of IL-1α and received no further therapy. With medical treatment, his congestive heart failure improved and a repeat echocardiogram four weeks later showed some improvement in his ventricular function although his ejection fraction had not returned to baseline.

In the control group, carboplatin 800 mg/m^2 produced the expected thrombocytopenia and granulocytopenia. The nadir platelet count occurred between 12-20 days after treatment and returned to normal 19-26 days after treatment. Four of the seven patients had grade IV thrombocytopenia with platelet counts less than 25,000/μL and these patients received platelet transfusions. The platelet count nadir in the other three patients were 56,000, 45,000, and 36,000/μL.

While data are still being collected and a formal statistical analysis has not been performed, a preliminary review of the data indicates that at the 0.03 mcg/kg dose level, IL-1α is not dramatically lessening the thrombocytopenia associated with carboplatin treatment. However, there are individual patients in the control group and in the other groups which show less thrombocytopenia with IL-1 treatment suggesting that IL-1α is capable of lessening thrombocytopenia. It remains to be seen whether this trend will be more consistently observed at the higher doses of IL-1α.

SIGNIFICANCE

This phase I study demonstrates that the combination of IL-1α and high dose carboplatin produced no new toxicities that were not observed in the phase I trial of IL-1α alone or with previous trials of carboplatin. Data are still being accumulated and at least ten more patients are expected to be enrolled at the 0.3 μg/kg dose of IL-1α before it can be determined whether IL-1α can significantly limit the bone marrow suppression induced by high dose carboplatin.

PROPOSED COURSE

Five patients each in the 0.3 μg/kg IL-1α before and after groups will be enrolled. If a dose and schedule of IL-1 emerges that appears to significantly limit the bone marrow suppression induced by carboplatin, another group of patients will be enrolled; they will receive the optimal dose and schedule of IL-1α but receive escalating doses of carboplatin. Carboplatin doses will be escalated from 800 to 1200, then 1600, then 2000 mg until excessive hematologic toxicity is observed. Future possibilities include using this basic protocol framework to test other hematopoietic growth factors in combination with IL-1 and high dose carboplatin such as G-CSF or IL-3, and when they become available, IL-6 or stem cell factor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09366-01 CRB

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.)*
Phase II Trial of IL-1α + Indomethacin in Melanoma Patients

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

PI:	J. W. Smith II	Medical Officer	BRMP, NCI
Others:	D. L. Longo	Associate Director	OAD, BRMP, NCI
	S. P. Creekmore	Chief	BRB, NCI
	R. G. Fenton	Expert	BRMP, NCI
	W. H. Sharfman	Expert	BRMP, NCI

See next page

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba); Cancer Therapy Evaluation Program, NCI, Bethesda, MD (M. Sznol, L. Miller)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

IL-1α has antiproliferative activity against human melanoma cell lines, causes murine melanoma tumors to shrink in vivo, can induce interferons and interleukin-2 which have activity in melanoma, and can enhance the activity of cytotoxic T cells and other effector cells. Animal studies suggested that indomethacin increased the antitumor effects of IL-1. For these reasons, we designed this phase II study in melanoma patients to determine the anti-tumor activity of IL-1α plus indomethacin using the maximum tolerated dose of IL-1α from our earlier phase I trial. A total of 36 patients have entered the study, and 32 are evaluable for response. No patient with predominately visceral metastases responded to treatment. One patient with non-visceral metastases had a partial response and one patient had a minor response. These results indicate that IL-1α has a low level of activity in melanoma. In an effort to enhance this activity, we are planning a follow-up clinical trial that combines etoposide (VP-16) with this regimen based on preclinical studies that have demonstrated synergy of the combination.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Medical Officer	BRMP, NCI
Dan L. Longo	Associate Director	OAD, BRMP, NCI
Stephen P. Creekmore	Chief	BRB, NCI
Robert G. Fenton	Expert	BRMP, NCI
William H. Sharfman	Expert	BRMP, NCI
John E. Janik	Expert	BRMP, NCI
Barry L. Gause	Medical Officer	BRMP, NCI
Jon T. Holmlund	Medical Officer	BRMP, NCI
Brendan D. Curti	Staff Fellow	BRMP, NCI

OBJECTIVE

To determine the phase II response rate of IL-1α plus indomethacin administered to patients with malignant melanoma at the maximum tolerated dose defined by a previous phase I trial.

METHODS EMPLOYED

Patients were eligible for this trial if they had a histologically confirmed diagnosis of malignant melanoma that was not curable by surgery. Patients were required to have a Karnofsky performance status ≥ than 70%, life expectancy of at least 3 months, measurable disease, and adequate physiological function. Prior chemotherapy was permitted and one prior course of immunotherapy was permitted. Patients with ocular melanoma were not excluded.

After stratification for the predominant site of metastatic disease, patients were treated with IL-1α 0.1 μg/kg IV over 15 minutes once daily for 7 days. Indomethacin 50 mg p.o. or p.r. was given starting 2 hours before the first dose and continuing every 8 hours during IL-1 treatment. Treatment was given as an inpatient and repeated once after a two-week rest. Three weeks after the last treatment, patients were reevaluated and responding patients continued therapy.

Fourteen patients had predominantly visceral metastases and twenty-two patients had predominantly non-visceral metastases. More than half of the patients had no prior therapy.

MAJOR FINDINGS

None of the 14 patients with visceral metastases responded to therapy. One patient had stable disease with treatment and has had no change in the size of his tumor for more than 6 months off therapy. Another patient in this group has had a shrinkage in his large liver metastases, but does not yet meet the criteria for partial response and continues on treatment. In the patients with predominantly non-visceral metastases, one patient has had a partial response (greater than 80% reduction in the size of his metastatic lesions) that has

lasted more than 10 months. Another patient in this group has had a minor response to therapy and continues on treatment. Another patient had a greater than 50% shrinkage in a supraclavicular lymph node but no change in a pelvic lymph node and is counted as having stable disease. Three other patients had individual metastatic lesions shrink but had other lesions progress and are recorded as having had progressive disease.

Toxicity was similar to that observed in the phase I trial of IL-1α and consisted of chills, fever, headache, nausea, vomiting and myalgia which were grade II or less. Grade III hypotension requiring IV pressors occurred in one-third of the patients and was limited to the first dose in all but three patients who required pressors, also with their second dose. All toxicities improved with repeated doses of IL-1. One patient developed bradycardia and a junctional rhythm during gastrointestinal discomfort and is presumed to have had excessive vagal tone. This patient responded rapidly to one dose of IV atropine. He also had hypotension requiring pressors. There was no evidence for myocardial infarction and the patient was retreated one week later with IL-1α without any further episodes of junctional rhythm. Another patient, a 77 year old woman with ocular melanoma, experienced shortness of breath after her last treatment with IL-1α. Echocardiogram and EKG were consistent with a myocardial infarction and congestive heart failure. The patient's congestive heart failure improved with standard therapy and follow-up echocardiogram two weeks later showed a slight improvement in her ejection fraction to 40%.

SIGNIFICANCE

This phase II study of IL-1α plus indomethacin in patients with melanoma demonstrates that this treatment has a response rate of less than 20% in patients with predominantly visceral metastases. In the group of patients with predominantly non-visceral metastases, the response rate is 6%. If no additional partial responses are observed in a total of 25 patients, then the response rate in patients with non-visceral metastases can be described with statistical confidences as less than 20%.

PROPOSED COURSE

The accrual goal for patients with visceral metastases has been met and no further patients will be entered in that group because no responses were seen in the first 14 patients. The accrual goal for patients with non-visceral metastases will be 25 patients if no additional patients respond. If additional patients respond to therapy, up to 35 patients will be enrolled.

The low level of antitumor activity observed with IL-1α treatment has caused us to consider combining it with another agent that might enhance the response rate in patients with melanoma. Preclinical studies by N. Usui at the BRMP have shown that etoposide (VP-16) can synergise with IL-1α in melanoma cell lines as well as in in vivo tumor models. Based on this observation, we have amended the trial to treat patients with melanoma in both visceral and non-visceral categories with a combination of IL-1α, indomethacin, and oral VP-16. The VP-16 will be given

orally because several clinical trials have shown that prolonged administration of VP-16 results in greater antitumor activity. The toxicity of these two agents are not overlapping; in fact, the IL-1α may ameliorate the dose-limiting toxicity of oral VP-16 which is myelosuppression. This amendment has been approved and accrual is expected to begin in the near future.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09367-01 CRB

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.)*
Evaluation of Activated Killer T Cells (T-AK) With Anti-CD3 and IL-2

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:	K. C. Conlon	Senior Staff Fellow	CRB, NCI
	J. E. Janik	Expert	CRB, NCI
	W. H. Sharfman	Expert	CRB, NCI
	J. W. Smith II	Medical Officer	CRB, NCI
	B. D. Curti	Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc./DynCorp, Frederick, MD (W. J. Urba, A. C. Ochoa)

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

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

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

Animal models have demonstrated that T cells stimulated with both anti-CD3 and interleukin-2 (IL-2) are effective against a variety of LAK and IL-2 resistant tumor lines. In addition, these activated killer T cells (T-AK) traffic in greater numbers to tumor nodules than LAK cells. Based on these findings, and a Phase I study of anti-CD3 alone in patients with advanced malignancies, a strategy was devised to stimulate autologous lymphocytes ex vivo with anti-CD3 followed by IL-2 in vivo. To accomplish this, peripheral blood lymphocytes are obtained from patients by leukapheresis. These cells are stimulated in the lab with anti-CD3 for 12-16 hours. They are then transferred back to the patient on the following day and IL-2 is begun. The number of cells administered and the time course and dose of IL-2 varies with the assigned treatment cohort. Five cohorts have been completed with additional ones planned. An atypical lymphocytosis with a concurrent anion gap metabolic acidosis and coagulopathy as well as the previously reported renal, hepatic and neurologic toxicities with IL-2 were seen. The acidosis was severe enough to require mechanical ventilation and dialysis in one patient. No other grade IV pulmonary toxicities related directly to this protocol have been seen. Of the 30 patients treated thus far, there has been one partial and one minimal response.

PROJECT DESCRIPTION

PERSONNEL

Dan L. Longo	Associate Director	OAD, BRMP, NCI
Kevin C. Conlon	Senior Staff Fellow	CRB, NCI
John E. Janik	Expert	CRB, NCI
William H. Sharfman	Expert	CRB, NCI
John W. Smith II	Medical Officer	CRB, NCI
Brendan D. Curti	Staff Fellow	CRB, NCI

OBJECTIVES

1. Determine the maximum number of T-AK cells that can be administered to patients with advanced cancer in combination with systemic IL-2.

2. Determine the toxicity of this regimen.

3. Measure the associated immunomodulatory effects.

4. Identify any tumor responses.

METHODS EMPLOYED

Patients with histologically confirmed metastatic and unresectable cancer are eligible. They must have an ECOG performance status of 0 or 1 and have adequate cardiac, pulmonary, hepatic, renal and bone marrow function.

The patients undergo up to 8 hours of leukapheresis on days 0 and 28 of their treatment cycle. After pheresis, the patients receive cyclophosphamide at a dose of 300 mg/m^2 IV. The leukapheresed cells are taken to the lab where lymphocytes are separated out with a Ficoll-Hypaque gradient. These lymphocytes are incubated overnight in serum free media with 10 ng/ml of anti-CD3. They are transferred back to the patient on the next morning and IL-2 is begun. The number of cells adoptively transferred in the first three cohorts was 5×10^9, 7.5×10^9 and 1×10^{10} cells respectively. These patients received IL-2 by continuous infusion at 3.0 mu/m^2/day and bolus at 1.5 mu/m^2/day. Their IL-2 continued for a total of 7 days after T-AK infusions on day 0 and 28 of the treatment cycle. Cohorts 4 and 5 received only continuous infusion IL-2 at doses of 3.0 mu/m^2/day and 1.5 mu/m^2/day respectively for 14 days. A cell dose of 7.5×10^9 was used for these cohorts, because this dose of cells caused the greatest peripheral lymphocytosis in the earlier cohorts. Patients achieving a partial response were eligible for additional cycles of treatment.

MAJOR FINDINGS

In the 5 cohorts treated thus far, a peripheral atypical lymphocytosis of up to 50,000 cells/mm^3 occurred 6-7 days after the administration of T-AK cells. This event was most prominent in the first and second cohorts. The last 3 cohorts had a much less pronounced lymphocytosis even though they were treated to the same degree of renal and liver toxicity as evidenced by similar increases in creatinine and bilirubin between cohorts. Flow cytometry showed that the circulating cells were CD3-positive T cells. Just before the appearance of these cells in the circulation, 14 out of 18 patients in the first three cohorts experienced the rapid onset of an anion gap metabolic acidosis. This was characterized by a low serum bicarbonate (< 10 mmol/L in 3 patients and <15 mmol/L in 8 others) and an increased serum lactate. One patient required mechanical ventilation and hemodialysis transiently for management of this problem. All other patients with bicarbonate values < 20 mmol/L were successfully treated with IV bicarbonate infusions. A coagulopathy was also seen in cohorts 1-3. This manifested as an increased PT and PTT which normalized when the patients' serum was mixed with normal serum. Factor assays showed deficiencies in factors VII, IX, X and antithrombin III. All patients corrected their coagulopathy after IL-2 was discontinued and with administration of vitamin K. There were no clinically significant bleeding episodes during treatment. Hypotension, mental status changes and decreased renal and hepatic function were observed as well. These sequelae were managed by dose reductions in IL-2 and appropriate supportive care measures. Cohorts 1 and 2 tolerated the greatest dose intensity of IL-2. Cohort 3 had the greatest amount of clinical toxicity and thus received the lowest percentage of the projected IL-2 dose. There has been one partial response in a patient with renal cancer and one minor response in one with hepatoblastoma.

SIGNIFICANCE

This regimen has demonstrated the successful adoptive transfer of anti-CD3 stimulated peripheral blood lymphocytes in conjunction with IL-2 and the expansion of a CD3-positive cell population in vivo. The rapid proliferation of these cells resulted in marked metabolic changes in some patients. The dose and method of administration (I bolus) intensity of IL-2 delivered appears to be more significant to cell growth than the initial dose of cells infused. Although cell expansion was achieved, antitumor effects were minimal in this phase I trial.

PROPOSED COURSE

Further treatment cohorts are planned to study the effect of other dosing intervals of IL-2 and T-AK cells on the immunologic, metabolic and antitumor effects of this protocol. Cyclophosphamide will be omitted from one cohort to determine if it is providing a significant immunomodulatory effect. In addition, some patients will receive IL-2 by bolus and continuous infusion without T-AK cells in order to better understand the contribution of the adoptive cell transfer to this regimen. Based on in vitro studies and on a previous clinical trial at the BRMP (8905), patients will be treated with low dose IL-2 alone or combined with IFNα. Cytokine will be administered subcutaneously, thereby allowing this therapy to be administered in the outpatient setting.

PUBLICATIONS

Curti B, Conlon K, Ochoa A, Urba W, Janik J, Sharfman W, Steis R, Smith II J, Holmlund J, Creekmore S, Fenton R, Sznol M, Miller L, Longo D. Phase I study of patients (pts) treated with bolus and infusional IL-2 and anti-CD3 stimulated cells (T-AK): Metabolic and coagulation disturbances. In: Perry MC, ed. Program/Proceedings American Society of Clinical Oncology. Houston: American Society of Clinical Oncology, 1991;10:211, abst. 706.

Ochoa AC, Conlon K, Loeffler C, Kopp W, Strobl S, Warner I, Smith K, Urba WJ, Longo DL. Activated T cells in the peripheral blood of patients receiving anti-CD3 stimulated lymphocytes and IL-2. Proc Am Assoc Cancer Res 1991;32:251, abst. 1494.

SUMMARY REPORT

ASSOCIATE DIRECTOR FOR THE DEVELOPMENT THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

NATIONAL CANCER INSTITUTE

October 1, 1990 - September 30, 1991

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 contract) 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, many of the administrative changes introduced during the previous year have been implemented in an effort to enhance the drug discovery and developmental programs. The anticancer screen is now fully operational. Over 10,000 tests were performed last year, and over 8,000 reports were sent to respective suppliers of agents. The backlog of compounds awaiting testing in the cancer screen was, therefore, markedly reduced allowing vigorous acquisition of new chemical entities to resume. In addition, pilot testing of a simplified approach to single concentration testing of crude natural product extracts was initiated in an effort to enhance the capacity for screening the diverse composition of the Natural Products Repository.

The Cancer Screening Committee has worked diligently over the past year to review the enormous number of tests on both the chemical entities and natural product extracts. Simultaneously, the criteria for referral of interesting potential leads has been established. In essence, agents or extracts are referred for further testing on the basis of tumor panel selectivity, potency, or antitumor activity identified through comparative studies based on agents with known interesting antitumor activity (i.e., the "COMPARE PROGRAM"). This remarkable computer-based selection process is accomplished by utilizing the "finger-print" of antitumor activity determined in the sixty cell-line anticancer screen. Agents with similar intracellular targets or mechanisms of action are identified through a series of algorithms. Subsequent correlation coefficients with the "seed" compound are provided. The agents that are identified by the Cancer Screening Committee as being of interest are assigned priority scores before submission to the Biological Evaluation Committee.

The Cancer Screening Committee has forwarded approximately 500 agents over the past year for review by the Cancer Biological Evaluation Committee. This interdisciplinary group of investigators reviews the referrals for uniqueness of chemical structure. Furthermore, related chemical entities are also discussed with additional consideration for agents selected for similar patterns of cytotoxicity or cytostasis by the COMPARE analysis. Each referral

is considered from a comprehensive perspective including the antitumor activity, uniqueness of structure or possible mechanism of antitumor activity, ease of procurement/synthesis, and formulation. Studies are subsequently planned and initiated to test in vivo antitumor activity. The Biological Evaluation Committee is essentially responsible for coordinating the entire spectrum of studies necessary for presentation to the Division of Cancer Treatment's Decision Network Committee (DNC).

Agents that receive approval for further development by the Decision Network Committee are subsequently followed by the Operating Committee and may be assigned to a Working Group to expedite the process of completing the necessary pharmacologic, toxicologic, and formulation research steps prior to submission of the INDA.

Major personnel changes over the past year include the appointment of Dr. Joseph Tomaszewski as the Chief of the Toxicology Branch. He subsequently recruited two individuals with drug development experience (Dr. Adeline Smith and Ms. Blaire Osborn) to the Toxicology Branch. Dr. Ravi Varma, an experienced natural products chemist, was recruited to the Drug Synthesis and Chemistry Branch to assist in the design and development of leads from the natural product drug discovery effort. Unfortunately, Dr. Ed Acton, who is the Deputy Branch Chief and Co-chairman of the Biological Evaluation Committee for DTP, has indicated that he will retire next month.

II. Program Accomplishments

A. Office of the Associate Director (OAD)

(1) Large Scale Procurement of Natural Products:

Significant activities related to large scale procurement of natural products with promising anticancer activity have demanded a considerable amount of time. In particular, the clinical responses observed with taxol have stimulated a substantial demand for enhanced procurement of this drug. The staff of DTP have been actively engaged in stimulating research activities related to renewable resources of taxol including semisynthesis and development of effective methods for extraction from cultivated taxus species. Coordination of activities with DTP's Natural Products Branch, additional federal agencies charged with responsibility for the natural resources (United States Department of Agriculture and the Bureau of Land Management), and the pharmaceutical industrial component (Bristol-Myers Squibb) has been a major responsibility for this office.

In addition to coordinating the plans for taxol procurement, the OAD has established a general plan for the large scale procurement of natural products that demonstrate interesting early clinical activity. DTP staff conducted a workshop attended by national experts in natural product drug discovery and development to specifically address the issue of procurement. Guidelines were developed that outline well-defined steps for investing progressively larger sums of money based on specified levels of antitumor activity. The potential mechanisms for effecting these procurements were identified, and a pilot plan for the procurement and scale-up of the camptothecins developed.

(2) Evaluation of the Cancer Screen and the Approach to Drug Discovery:

An evaluation of the data obtained from the cancer screen has been initiated. In addition, a series of workshops focused on data analysis and relative contributions of specific cell lines within the panel will occur over the next year. Additional emphasis on characterizing the biochemical and molecular characteristics of the current tumor panel has been initiated and will be examined in detail at a planned workshop. The description of new molecular targets for potential therapeutic exploitation will be the major topic of this workshop that has been planned for late 1991. Select targets will be discussed that might be incorporated into the existing screening capabilities for the NCI program.

Furthermore, the existing Natural Products Repository represents an enormous national resource for drug discovery. The difficulties in making this resource available to extramural drug discovery groups have been systematically approached over the past year. A careful plan for providing samples from this repository has been developed. A method for evaluation of the proposed uses of the facility has been described and the individual members of the reviewing committee selected. Finally, the announcement of this opportunity will be made in the early fall of 1991.

A plan for restructuring the Laboratory of Biological Chemistry has been initiated. The new focus of the laboratory will be strongly oriented towards a molecular targeted approach to drug discovery. Dr. Cysyk, the former laboratory chief, is redirecting his research interests to address the potential therapeutic advantages to modification of the extracellular matrix in the treatment of cancer. The Laboratory of Biological Chemistry will build on existing expertise in the areas of protein modification and packaging (e.g., control of protein secretion by the Golgi apparatus), and expand by recruiting additional expertise in molecular biology. Selection of the appropriate individual to lead this laboratory is critically important. Supported by the resources of the DTP, this laboratory is in a unique position and has the potential for being on the forefront of cancer therapeutic innovation.

(3) Antiviral Research:

The vigorous anti-HIV drug discovery and development program continues with high-volume screening. The Biological Evaluation Committee for AIDS has recently brought an exciting new class of agents to the Decision Network Committee. DTP has almost completed the necessary preclinical studies to introduce its first drug discovery product into clinical investigation. A potent analog (NSC 629243) of oxathiin carboxanilide will be ready for INDA submission by the end of 1991. Furthermore, a potent protease inhibitor, is also near completion of the preclinical studies with the anticipation of entering clinical investigation in early 1992.

The newly formed Antiviral Evaluations Branch has established collaborations with intramural and extramural investigators to perform the necessary secondary studies to define the basic mechanism of action(s) of new leads. Dr. McMahon in the Laboratory of Drug Discovery Research and Development is working closely with Drs. Bader and Weislow to enhance the scope of studies performed on interesting new leads.

In addition, the importance of close collaboration with medicinal chemists has recently been demonstrated (e.g., modifications of the parent oxathiin carboxanilide to potentially prevent metabolic inactivation). In DTP, the diversity of expertise exists which is clearly needed to optimize the new agents being developed for the clinic.

The Antiviral Evaluations Branch will undergo a program review over the next year to identify areas of potential expansion of secondary testing that will further complement the existing battery of investigations. Hopefully, the growth of this program will continue to provide interesting new modifications based on the initial leads from the successful antiviral screen.

B. Laboratory of Drug Discovery Research and Development (LDDRD)

The Laboratory of Drug Discovery Research and Development (LDDRD) originated within the Program Development Research Group organized by Dr. Michael Boyd during his leadership of DTP. This group of talented investigators with a highly focused research mission has been developed to expedite the discovery and development of high priority compounds discovered by the NCI screen. The group has a major commitment to investigate natural products as a potential source of new leads in the treatment or either cancer or AIDS. In addition, investigators within the LDDRD are committed to investigating secondary studies necessary to define the mechanism of action, pharmacology, and metabolism of new leads.

The LDDRD is organized into three sections including: the Cell Biology, Biochemistry and Experimental Therapeutics Section; the Natural Products Chemistry Section; and the Analytical Chemistry, Pharmacokinetics, and Metabolism Section. This laboratory underwent an extensive review during this past year as part of the regular site visiting process. Many of the site visitors suggestions have been implemented as the new laboratory emerges as a critical unit within DTP.

Prototype antiviral agents are being utilized to develop and evaluate novel assay systems aimed at elucidating potential mechanisms of action of new compounds. The isolation and identification of several unique chemical entities have evolved from the screening efforts originating from this laboratory. A significant number of anti-HIV active extracts led to the association of phorbol receptor binding activity with AIDS antiviral activity. The discovery of prostratin led to this discovery with the subsequent identification of active triterpenes and xanthophylls. Anti-HIV alkaloids, dimeric alkaloids, and sulfated polysaccharides are under active investigation for careful chemical characterization. Structure-activity relationships are being defined, and methods for dereplication are under development to expedite the process of novel drug discovery.

Specific assays to define the mechanism of action of the promising new leads are complemented by *in vitro* cellular pharmacology to define the optimal chemical entity to take into clinical investigation. Additional development of *in vitro* and *in vivo* investigations of anticancer compounds is also underway in this laboratory. Specifically, new methods are being explored to determine cell survival following drug exposure, recovery of proliferative capacity and optimal *in vitro* chemotherapeutic schedules.

The application of molecular biologic approaches for defining the extent of human tumors in animal models is under investigation. Appropriate animal models to test new leads from the cancer and AIDS screens are also being examined. Biochemical and cellular modification of these agents are explored (e.g., increased intracellular accumulation of anti-HIV oxathiin derivatives has been achieved with the use of classical multiple drug resistance chemosensitizers, and conversion of inactive agents to active species by blocking esterase activity has been accomplished for several agents). The goal of the LDDRD is clearly to expedite the discovery and development of the best candidates for clinical investigation in the treatment of either cancer or AIDS.

C. Laboratory of Molecular Pharmacology (LMP)

The major goal of the Laboratory of Molecular Pharmacology (LMP) is to explore molecular and cellular targets that may be useful in developing new drugs or strategies in the treatment of cancer. Several important cancer drugs form stable complexes with the topoisomerases and the DNA with consequent cleavage of the DNA. The interaction of these complexes results in cellular cytotoxicity which is also related to interaction with DNA or RNA polymerases. The specific DNA localization of these interactions is different for each drug, and work is ongoing to define the difference between the responsive and resistant cells.

Microtubules are critically important cellular structures serving cell division and cytoskeletal functions. Novel agents that interact with tubulin inhibit mitosis and include: combretastatins, dolastatins, colchicine analogs, and halichondrins. This project focuses on the structure-activity relationships and the perturbations in the function of tubulin resulting from the interaction with these agents.

Alkylating agents have been connected to intercalating agents that result in selective DNA binding. Mustard and acridine moieties were connected via a straight hydrocarbon chain of variable length. These highly selective agents were potent in producing cytotoxic lesions, and are being examined in the human tumor cell line screen. In particular, the monofunctional acridine mustard with a connecting chain length of 3 is optimally restricted with respect to the range of chemical reactions that it would produce in the cell, and this compound is a potential candidate for drug development.

The ability of a cell to survive exposure to a drug is a function of the DNA repair efficiency. Preferential DNA repair occurs in transcriptionally-active areas of the genome. Interstrand crosslinks in specific genes are removed more rapidly in resistant cells than in the sensitive cells, whereas no difference is observed in the removal of intrastrand crosslinks. The mechanism of preferential DNA repair was investigated through the use of specific enzyme inhibitors. While the use of a single drug might not interfere with the preferential DNA repair, the use of the combination of two drugs (camptothecin and merbarone) did inhibit. Thus, the possibility that preferential DNA repair requires topoisomerase has been raised. The possible relationship between the efficiency of DNA repair and the origin of specific malignancies is also under investigation.

In addition to identifying the process of DNA repair as a target for novel chemotherapy, a description of the genes induced by DNA damage and their function is also potentially quite important. Since cell growth arrest in certain phases of the cell cycle may result from DNA damage, the events occurring during arrest may actually favor ultimate cell survival. The induction of gadd45 and gadd153 genes may be involved in the process of growth arrest following DNA damage. Other stress-response genes are also induced including the 15 different heat-shock genes, and may protect cells against subsequent DNA damage. Studies of the regulation of HIV gene expression in response to DNA damaging agents will also be examined in relation to the malignancies associated with AIDS.

Finally, the relationship between the syntheses of histone proteins and DNA are being examined as potential targets for the development of novel chemotherapeutic intervention. Since excess histone could be cytotoxic to a cell, the intricate balance of synthesis is important. The presumed site for maintaining this balance is mediated by the concentration of histones in the cytoplasmic soluble pool.

D. Laboratory of Medicinal Chemistry (LMC)

The Laboratory of Medicinal Chemistry (LMC) is involved with the discovery and development of both anticancer and anti-HIV agents. Cyclopentyl cytosine (CPE-C) was designed and developed by this group as an anticancer agent after initial work on other carbocyclic sugars (neplanocin A). CPE-C has an IC50 of 75 nM in MOLT-4 cells, and provides an intracellular CPE-CTP prolonged half-life in the range of 9 to 14 hours. Extensive collaboration between investigators in the LMC and other parts of the DCT have occurred over the past year as this agent is ready for initial clinical trial.

The disposition and pharmacokinetics of CPE-C administered by continuous intravenous infusion in dogs were studied with the Toxicology Branch in DTP to prepare the data for INDA filing. Additional work by the LMC has provided a very sensitive analytical method allowing quantitation to the 0.01 uM range, thus hopefully, permitting Clinical Oncology Program (COP) investigators to determine plasma concentrations during the initial phase I trial.

Additional nucleosides were evaluated for antitumor and antiviral activities. Zebularine, a 2-oxopyrimidine riboside, has demonstrated in vivo activity against L1210 and certain viruses. Furthermore, zebularine has been demonstrated to be a potent inhibitor of cytosine deaminase. Psicofuranine A, a noncytotoxic nucleoside in vitro has demonstrated good activity against several viruses including RSV, measles, and parainfluenza. Finally, 3-deazaneplanocin A, synthesized several years ago by the LMC, continues to be of interest as additional observations of its antiviral activity against African swine flu, measles in vitro, and the Ebola virus in vivo have been made.

Potential inhibitors of both tyrosine kinase and protein kinase C are being evaluated that might ultimately permit modulation of the transduction events leading to malignancy. Phenazocine inhibits the autophosporylation of the EGF receptor similar to that of erbstatin. In addition, polyhydroxylated cinnamamide derivatives are being synthesized as potential inhibitors of tyrosine kinase. Finally, a hydrolytically stable analog of O-phosphotyrosine

has been developed that may interfere with the action of tyrosine kinase by interfering with the recognition of the endogenous substrates.

Inosine monophosphate is important in the anti-HIV activity of dideoxyinosine (ddI). Investigators from the LMC have demonstrated that the inhibition of IMP dehydrogenase by ribavirin increases the anti-HIV activity of ddI fourfold. Phosphorylation is very important for the anti-HIV activity.

Other novel leads for the therapy of AIDS include: dideoxyapiose nucleosides; substituted purines; and polypeptides designed and synthesized as inhibitors to various regions of the HIV protease using the sense/antisense concept. In addition to the search for novel therapeutic approaches, investigators in the LMC are continuously evaluating newer analytical methods (e.g., continuous-flow fast atom bombardment mass spectrometry combined with on-line capillary liquid chromatography).

E. Laboratory of Biological Chemistry (LBC)

The Laboratory of Biological Chemistry (LBC) has investigated several new potential therapeutic targets for the treatment of both cancer and AIDS. The investigators have explored several important protein modification reactions that could have an impact on the intracellular location and function 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. The post-translational protein acylation with myristic acid (i.e., N-myristoylation), isoprenoids (i.e., isoprenylation), and retinoic acid (i.e., retinoylation) are being examined as potential targets for intervention. In the case of N-myristoylation, blockage of this process could interfere with both cellular transformation and viral replication. N-myristoyl transferase has been purified, and the active enzyme appears to be a dimer made up of a mixture of 4-5 different isozymes. A new compound N-myristoyl tetrazole has been demonstrated to inhibit HIV syncytia formation and protein N-myristoylation in tissue culture cells. The exact interaction with myristoylation is under investigation.

The importance of retinoic acid as a differentiation promoter has been demonstrated clinically. The cellular mechanism for this phenomenon is under investigation. In this laboratory, the formation of a covalent bond between retinoic acid and proteins has been demonstrated. In the leukemic cell line HL60, there are at least 20 retinoylated proteins localized in the nucleus. The retinoic acid probably links to protein via the formation of a thioester bond. Furthermore, investigators in this laboratory have demonstrated that these retinoylated proteins are CAMP-binding proteins. Several of these retinoylated proteins appear to react with regulatory subunits of CAMP-dependent protein kinase. Therefore, the interaction of these retinoylated proteins could play key roles in either cellular transformation or differentiation.

Another important family of proteins (specifically, the small GTP-binding proteins) that may have an impact on cellular transformation and growth, protein synthesis and processing, targeting of membrane vesicles and organelles, and activation of second messenger systems (e.g.phospholipase C) have been a major focus of the laboratory. ADP-ribosylation factor, ARF, is one of the key proteins that has been investigated. Recent data have

demonstrated that this small GTP-binding protein is essential in the maintenance and functioning of the cellular Golgi apparatus, and it can be directly activated by the anti-metastatic gene nm23. In addition to exploring mechanisms for utilizing this new information to design drugs that might interfere with cellular malignant transformation, the processes of invasion and metastasis may also be amenable to modification.

The control of de novo and salvage synthetic routes for pyrimidines has been a long-standing interest of this laboratory. The transfer of Dr. Cysyk to the Laboratory of Medicinal Chemistry will permit an evaluation of the feasibility of the modifications in these nucleoside control pathways to have a role in cancer therapy. These efforts will complement experiments currently under progress in the laboratories of Drs. Driscoll and Johns. Finally, Dr. Cysyk will provide additional biological expertise to the LMC, and this will be consistent with the recommendations of the last site visit to that specific laboratory.

F. Biological Testing Branch (BTB)

The Biological Testing Branch (BTB) is responsible for the implementation of the in vitro cancer and AIDS screens. In addition, this branch is responsible for the in vitro and in vivo confirmatory studies.

Over the past year, the targets for anticancer and anti-HIV screening have been met. There were over 20,000 tests performed in the cancer screen, and approximately 40,000 tests completed in the anti-HIV screen respectively. The plan to undertake a formalized approach to follow-up in vitro studies is being developed. The Cancer Screening Committee of DTP has done a superb job of reviewing the screening data, and is currently embarking on a plan to analyze the results of the cell line constituents. The in vitro screening program has referred over 500 agents to the Biological Evaluations Committee of DTP based primarily on the screening data.

The in vivo program has been successful in providing subcutaneously implanted xenographs for approximately 90% of the tumor cell lines in the main cancer screening panel. Furthermore, collaborative arrangements have been made with specific investigators from outside of the NCI who will participate in the critical in vivo assessment of either anticancer or antiviral activity.

Over the past several months, the staff of the BTB has been working on a promising new in vivo assay system that would both expedite obtaining early efficacy data and would require much less material to initiate the in vivo assessment. Additional in vivo testing with the established subcutaneous xenograft models would also be pursued to confirm efficacy. Furthermore, this branch is attempting to develop innovative in vivo models that would more closely resemble the human malignant states. Consequently, contract efforts are currently focusing on the development of appropriate in vivo models for both prostate and breast cancers. In addition, specific efforts are also underway to develop additional prostate and breast cancer cell lines that will be amenable to testing in the cancer screen or some variant thereof, and there are approximately 5 to 6 breast cancer cell lines that hopefully will be ready for pilot screening in the near future.

In addition to the routine confirmatory testing of anti-HIV agents, there now are additional systems operational for evaluating a syncytium assay and the production of p24 antigen. Models for in vivo testing of novel anti-HIV testing are under development both within DTP and through collaboration with NIAID.

Currently, the BTB is recruiting for a scientist to coordinate the in vivo testing program. The bulk of the routine testing for cancer agents is conducted through the extramural contract mechanism. The developmental aspects of the in vivo program involves both intramural and extramural efforts. In February, 1992 a panel of extramural experts will be convened to review the operational methods of the entire in vivo program. Planning for this review has been initiated.

The BTB is also responsible for the animal production facilities which are managed through extramural contracts. This enormous animal production program actually supplies extramural and intramural scientists with a very cost-effective high-quality source of experimental animals. In addition, the BTB also operates a tumor bank to provided investigators with a diverse array of tumor cell lines including those that are part of the NCI cancer screen.

G. Drug Synthesis and Chemistry Branch (DSCB)

Over the past year, the Drug Synthesis and Chemistry Branch (DS&CB) procured 10,600 submissions through its acquisition contractors for the cancer screen. This included approximately 600 identified natural products and 10,000 defined chemical entities. This branch is also responsible for coordinating the flow of compounds through each of the primary screens (i.e., 200/week into both the cancer and AIDS screens).

This branch was responsible for managing the chemical synthesis contracts that provided 93 compounds to the program for further testing. The radiolabelled syntheses of 9-aminocamptothecin, 9-methoxyellipticinium, and "Uniroyal Junior" were accomplished over the past year, and the labelled syntheses of taxol and Dolastatin 10 were initiated.

Efforts to identify structure activity relationships and effective congeners for both cancer and AIDS agents have been initiated. A new synthetic effort to expand on natural product leads has recently begun with plans to develop an accessible topological model of taxol.

H. Grants and Contracts Operations Branch (GCOB)

The staff of the Grants and Contracts Operations Branch (GCOB) were responsible for the administrative management of 315 grants in the area of biochemistry and pharmacology which represented over $64,000,000 in financial research support. In addition, the staff participated scientifically in cooperative agreements for 22 drug discovery groups in the National Cooperative Drug Discovery Groups Program (NCDDG). These cooperative agreements have resulted in five agents reaching clinical investigation since 1983, and many excellent new groups have recently begun their search for novel therapeutic products.

The staff of the GCOB has participated in an extensive review of the contract program in support of DTP's drug discovery and development program. The attempts over the past year to consolidate contracts that had overlapping workscopes will result in savings of funds and staff administrative time and effort.

I. Information Technology Branch (ITB)

Modifications to the COMPARE program have continued to enhance the utility of this system for identifying agents with similar mechanisms of action. This *in vitro* data pattern matching system has provided leads to identification of new tubulin interactive agents, a new class of topoisomerase II interactive agent, and several new potential antimetabolites suspected of inhibiting enzymes of de novo pyrimidine biosynthesis.

Enhanced computer support for the entire drug discovery and development program was procured over the past year in large part by utilizing money saved by the system that had been implemented several years ago (i.e., the financial savings resulting from the use of the VAX 8820 made it possible to purchase the VAX 9210). Furthermore, the utilization of the previous system (VAX 8820) was at the maximum, and a careful study demonstrated that additional computer support was essential for the viability of the program.

The current system should be sufficient at least for the next two to three years. There are additional critical functions now being performed by the computer including: (1) all supplier reports for cancer and AIDS screens; (2) secondary test results (e.g., results syncytial assay, p24 production, and *in vivo* testing are now provided to the supplier; (3) a new tracking system (DTP Retrieval System) for all agents passed by DNC and being followed by the Operating Committees are on computer; (4) DTP MENU SYSTEM is now available for general use; (5) three subsystems to facilitate natural products crude extract fractionation is now available; and (6) major modifications of the Pharmaceutical Data System (PDS) have been implemented providing substantial versatility and added accountability.

J. Natural Products Branch (NPB)

The staff of the Natural Products Branch (NPB) are responsible for the acquisition of diverse crude natural products, their extraction, and organized testing in the cancer and AIDS screens. In addition, the staff is responsible for conducting research in following up interesting preclinical and clinical natural product leads. Extensive efforts have been expended in the large scale procurement of taxol for clinical investigation.

The current inventory of the Natural Products Repository includes approximately 50,000 extracts, and over 31,000 have been tested for anti-HIV activity. In addition, approximately 6,000 extracts have been tested in the cancer screen. Accelerated protocols have been developed to potentially expedite the cancer screening.

While prior efforts in natural product drug discovery had focussed on land-based bacteria, actinomyces and fungi, DTP has taken a course looking for novel sources of organisms. A novel class of compounds (i.e., glycosulfolipids) exhibiting *in vitro* AIDS antiviral activity was isolated

from the cyanobacteria (blue-green algae). The NCI is also investigating many unusual fungi and marine organisms.

The plant program of the NPB has been tremendously successful in obtaining numerous specimens from diverse environments from Africa (Madagascar), Latin and South America, and the Indonesian areas. Two novel alkaloids exhibiting in vitro anti-HIV activity have been isolated from a Cameroon plant by LDDRD chemists and are being further investigated.

Furthermore, the demonstration of anticancer activity with either Taxol or Topotecan (a derivative of camptothecin) has provided a major commitment to assist in the large scale procurement of these natural products. There have been numerous efforts including: surveying the content of varied plant species; educational sessions to convince other scientists that renewable resources were preferable to continued reliance on the taxus bark; consulting on the feasibility of improved methods of taxol extraction from the bark and alternative sources (e.g., cultivated taxus plants); participation in planning sessions to avoid a similar supply crisis for future natural products of clinical interest.

Extensive collaborative efforts have been established including: an agreement with the Kunming Institute of Botany; Natural Products Research Institute in Seoul, Korea; Institute of Medicinal Plants in Cameroon; and the Tel Aviv University in the study of Red Sea marine organisms. Numerous efforts have also resulted in collaborative arrangements with additional African and Latin American sources of natural products. The DTP/NPB staff were very instrumental in organizing the international workshop this spring entitled "Drug Discovery, Biological Diversity, and Economic Growth." Interagency agreements to collaborate on projects that would potentially benefit scientists and the third world area in possession of the natural products were extensively discussed between the National Science Foundation, the Agency for International Development, the National Cancer Institute, and the Fogerty International Center.

The Natural Products Repository represents a national resource that will be systematically made available to highly competent natural product chemists that are committed to drug discovery. Significant work has been accomplished over the past year to insure that this resource is handled in a highly responsible manner in order to avoid squandering of these incredibly valuable samples.

Finally, the NPB was extensively involved with the issue of scale-up production and procurement of natural products that had demonstrated evidence of promising clinical activity. While these efforts were mainly driven by the difficulty in procuring sufficient supplies of taxol for patients, specific recommendations were made by extramural experts that hopefully will avoid similar supply crises in the future.

K. Pharmacology Branch (PB)

The staff of the Pharmacology Branch (PB) have consolidated the analytical methods development contracts for cancer and AIDS. Since the approach to methods development is quite similar with an overlapping workscope, this consolidation should result in financial and staff time conservation. In

addition to initial pharmacokinetics studies, the staff has been responsible for executing detailed pharmacologic studies in conjunction with efficacy evaluations.

Approximately six anticancer classes of agent and eight novel anti-HIV compounds were evaluated over the past year. Several representative examples include: (1) Extensive pharmacologic studies were completed on 9-aminocamptothecin (9AC) with definition of the importance of maintenance of a plasma concentration in a specific range (10 nM range) for a time period approximating 72 hrs. in an in vivo model with a relatively rapid tumor growth profile; (2) A specific assay method for the 9AC was developed that permits in vivo quantitation of the closed (active anticancer form) and open ringed forms of the compound; and (3) Dr. Liotta's CAI compound was demonstrated to produce readily detected plasma concentrations when administered orally.

Combinations of agents have been investigated within the branch including studies to elucidate the optimal agents to be added to taxol, and the combination of BCNU and potential modulators of its anticancer activity (O-6-methyl and O-6-benzylguanine).

L. Toxicology Branch (TB)

The staff of the Toxicology Branch (TB) are responsible for determining the end-organ toxicities and additionally establishing the dose-responsiveness and schedule dependency of these adverse effects. The plasma drug concentrations are also correlated with the safety and toxicity and the in vitro or in vivo efficacy. Safe starting doses are established, and the specific toxicities that may be encountered in patients are predicted.

Over the past year, three new antineoplastic agents and four new anti-HIV agents moved into initial toxicology studies (DN stage IIA testing). One of the agents (Merck L651582) moved from DN IIA to DN IIB stage (ie., moved into the full toxicologic studies necessary for INDA filing). Another agent 9-aminocamptothecin is beginning IIB shortly.

One of the five anti-HIV drugs that were actually in stage DN IIA last year was dropped in favor of a second generation agent that appeared to be a better clinical candidate (oxathiin carboxanilide). A second agent was dropped for other reasons, and three remain under DN IIA investigation.

At the DN IIB level, the Merck L651582 and penclomedine will soon be completed. The 8-chloro cyclic AMP had produced nephrotoxicity and additional schedule dependency data is being accrued. There are two anti-HIV agents in DN IIB testing (i.e., a protease inhibitor and Uniroyal Jr.). Finally, the TB has evaluated toxicity encountered with several combinations of novel agents that were of high interest to the Cancer Therapy Evaluation Program (CTEP) (e.g.,carboplatin and BSO).

Innovative protocols to evaluate in vitro toxicity are being pursued (e.g. comparative marrow toxicity). Additional data has also been accrued utilizing the experimental model for evaluating the in vivo availability of anti-HIV agents. Since there is a real need for a valid in vivo model to determine the efficacy of anti-HIV agents, the TB staff have developed an experimental protocol to test serum from animals treated with potential anti-HIV agents.

This serum is then utilized in the in vitro assay for AIDS which gives an assessment of the potential efficacy of the agent in vivo. The results to date have demonstrated that 12/18 drugs active in the initial in vitro assay are found to be active following administration to an animal with subsequent in vitro testing of serum obtained from the animal. These evaluations may indicate that effective concentrations of the agents were achievable in vivo, and correlations with the subsequent clinical data will be required to fully assess the validity of this model.

M. Pharmaceutical Resources Branch (PRB)

The staff of the Pharmaceutical Resources Branch (PRB) is responsible for large scale chemical synthesis and analysis, formulation research, pharmaceutical manufacturing, shelf-life surveillance, product storage and distribution. During the past year, the PRB produced over 500,000 injectable units and approximately 50,000 oral dosage forms for clinical distribution. Staff also prepared an update of the manual NCI Investigational Drugs: Pharmaceutical Data 1990 (NIH Publication No. 91-2141).

During the past year PRB staff were involved with the development of emulsions and microdispersions for intravenous administration. Innovative approaches to difficult formulation problems have been pursued (e.g., 9-aminocamptothecin), but additional efforts in this area are clearly needed. Therefore, a recruitment effort for a physical pharmacist has been initiated in accord with recent recommendations of consultants obtained for the branch.

N. Antiviral Evaluations Branch (AEB)

The primary responsibility of the Antiviral Evaluations Branch (AEB) is the supervision of the AIDS antiviral screen. This responsibility includes participation in the technical features of the laboratory studies, monitoring data, interpretation of results from the screen, and the transmission of the results to the suppliers and the appropriate staff of DTP. In addition to the primary responsibility of screening, this branch also attempts to define the mechanism of action of agents by working in close collaboration with both intramural and extramural scientists.

Changes in the technical features of the screen have improved the efficiency and reliability of the antiviral tests. During the past year over 95 % of all tests passed strict quality control standards. Consequently, the real capacity of the screen has increased, and over 20,000 unique synthetic compounds and biological extracts were tested in the past year. Also, close attention to the amount of infectious virus employed in the tests has expanded the sensitivity of the screen to detect materials active at any stage of the HIV reproductive cycle.

Active compounds of either unique structure or potent antiviral activity are examined in detail. Inhibition of virus reproductive activity and protection of cells from HIV killing are confirmed in additional tests. The range of action is examined by testing these compounds against several HIV-1, HIV-2 and a murine retrovirus. Methods for testing such compounds against AZT-resistant HIV strains, and in combination with AZT are under development. The compounds are also tested in several T cell lines, fresh peripheral blood leukocytes, and other cells. During the first year of operation of the branch, significant

collaborations have been developed to expand the preclinical evaluation capacity for the new leads discovered in the primary screen.

Over fifteen new classes of compounds with antiviral activity have been discovered to inhibit HIV in the screen. One of these discoveries oxathiin carboxanilide (NSC 615985) and a more potent analog (NSC 629243) were demonstrated to interfere with virus reproduction at a stage after virus binding to the cellular receptor but prior to reverse transcriptase. Apparent inhibition of the fusion of the virion and the cellular membranes required during early infection is prevented by the oxathiin carboxanilide. In addition, other interesting agents with different modes of action are under active investigation by the program. The staff of the AEB have been active in presenting results of this work at numerous national and international meetings.

III. Conclusion

The Developmental Therapeutics Program is a complex entity with extensive intramural and extramural resources devoted to the discovery and subsequent development of novel agents for the treatment of either cancer or AIDS. While the overall program is coordinated through the Office of the Associate Director, there is close communication with all aspects of the Division. The current progress of potential new clinical drug candidates through the DCT/NCI Decision Network process is summarized in Table 1. Finally, the membership of the Working Groups that are responsible for expediting the developmental progress of the high priority agents is provided in Table 2.

Table 1

COMPOUNDS THAT PASSED DECISION NETWORK (4/1/90 - 3/31/91)

NSC Number	Name	Compound Type*
	Decision Network IIA	
	Antineoplastic	
376128	Dolastatin 10	NP
630176D	Discreet	NP
642492	Fumagillin analog (AGM-1470)	SS
	Anti-HIV	
640441D	Discreet	S
	Antineoplastic	
609974	Autocrine motility factor	S
	Anti-HIV	
629243	Uniroyal, Jr.	S
	Decision Network III	
	Antineoplastic	
339555	Bryostatin 1	NP
375575	Cyclopentenylcytosine	S
639179D-181D	Discreet	B
639182D	Discreet	B
639184D	Discreet	B
639185D	Discreet	B
639186	ICI-D1694	S
	Decision Network IV	
	Antineoplastic	
032065	Hydroxyurea	S
105014	2-Chlorodeoxyadenosine	S
357885	Anthrapyrazole (CI941)	S
624239	PEG-L-Asparaginase	SS

*S = synthetic
NP = natural product
B = biologic
SS = semisynthetic (natural product modified synthetically)

Table 2

Working Groups of the Operating Committees

A. Cancer Drug Development

Cancer Drug	Chairperson
Cyclopentyl Cytosine	Dr. Tomaszewski
8-Chloro-cAMP	Dr. Zaharko
Camptothecins	Dr. Plowman
Ellipticinium	Dr. Boyd
609974D	Dr. Curt
630176D (Fugisawa)	Dr. Lednicer
CC-1065 Analogs	Ms. Osborn
Fumagillin Analogue (AGM-1470)	Dr. Schepartz

B. Antiviral Drug Development

Antiviral Drug	Chairperson
624231D	Dr. Covey
625487D	Dr. Covey
629243D	Dr. Boyd
640441D	Dr. Tomaszewski
638352D	Dr. Clanton

ANNUAL REPORT OF THE LABORATORY OF DRUG DISCOVERY RESEARCH AND DEVELOPMENT

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

INTRODUCTION

The LDDRD is an unprecedented new NCI/NIH intramural organizational entity, having a highly applied, programmatically-oriented research mission within DTP, DCT, NCI. The LDDRD, only recently (August, 1990) constituted formally as a Laboratory, has its origins from the DTP Program Development Research Group (PDRG). The PDRG was a consortium of investigators initially recruited and organized by Dr. Boyd within the Office of the Associate Director, DTP, to provide direct research and development support for the implementation of DTP's new antitumor and anti-HIV drug screens, and to provide for an enhanced intramural emphasis upon natural products drug discovery research and development. The LDDRD is organized into three sections, including the Cell Biology, Biochemistry and Experimental Therapeutics Section (CBBETS), the Natural Products Chemistry Section (NPCS), and the Analytical Chemistry, Pharmacokinetics and Metabolism Section (ACPMS). The research emphasis of the sections and the individual investigator projects therein are collectively focused upon the overall programmatic mission of the LDDRD: "to plan and conduct a program of basic and applied research for antitumor and antiviral drug discovery and development from plant, marine and microbial natural products, and from selected new synthetic or semisynthetic chemical classes identified by the NCI screens".

LDDRD RESEARCH PROGRAM

LDDRD (PDRG) investigators have played numerous pivotal roles in the conceptual and technical development of both the NCI's *in vitro* disease oriented primary antitumor screen and NCI's primary anti-HIV screen. LDDRD investigators continue to be centrally involved in the further refinement and evolution of the primary screens, as well as for the development and application of more specialized methodologies for the detailed biological evaluation of new active agents. There is increasing focus upon the application of the new screens to natural products drug discovery. Further, specialized facilities are being developed, appropriate expertise recruited, and research activities initiated, to support the detailed and comprehensive preclinical pharmacologic, toxicologic and experimental therapeutic evaluation of selected high priority lead compounds and their prioritization for INDA-directed preclinical drug development and clinical testing. Some highlights of LDDRD research contributions during the past year are provided in the following.

Development and Applications of Mechanism-oriented Assays and Other Assays for "Stage II" Evaluation of New Lead Compounds Identified by the NCI Primary Anti-HIV Screen

Prototype antiviral agents are being used to develop and evaluate novel assay systems aimed at elucidating potential mechanisms of action of new compounds

identified by the NCI primary anti-HIV screens. The assays define specific stages in the virus life cycle and include a diverse set of biochemical endpoints. Prioritization of new lead compounds is facilitated through the derivation of a wider variety of in vitro test data than available from the primary screen.

Development and Applications of In Vitro Methods of Anticancer Drug Screening and Stage II Evaluation

A variety of alternative in vitro antitumor assays are being developed and comparatively evaluated with a view toward possible applications in primary (Stage I) screening or to help prioritize (Stage II) new agents for further study. Specifically, new methods are being explored for determination of cell survivorship of drug treatment; recovery of proliferative capacity following drug treatment; kinetics of drug action; pulse duration effects on cellular responses; and optimization of in vitro chemotherapeutic schedules. Moreover, methods for assessment of the above not only in monolayer culture but also in single-cell suspensions, weakly adherent lines, floating aggregates and spheroids are being pursued.

Development, Calibration and Application of Agar-Based Assay Systems for Detailed In Vitro Evaluation of Priority Compounds

We have evaluated the potential utility of agar assays to: a) confirm differential in vitro test results using the selected cell lines in an alternate culture format, b) to verify in vivo drug efficacy (or lack thereof) by in vitro assay of tumor xenografts treated in vivo, and c) to assess certain kinds of synthetic and natural products which may give false positive tests in fluid monolayer culture assays. We have developed an under-agar monolayer culture system that appears well-suited for accurate drug sensitivity determinations of compounds which liberate cells from fluid monolayer culture systems due to chemical and/or physical properties of the test material. In addition, we are refining in vitro soft agar colony formation assays to a) quantitate the clonal fraction and relative mass of cells which survive treatment in the primary in vitro and in vivo anti-cancer drug screens, and b) permit isolation and subsequent characterization of drug-resistant cell populations.

In Vitro Cellular Pharmacology of New Anti-HIV and Antitumor Drugs

Studies of a novel oxathiin carboxanilide identified by the primary NCI anti-HIV screen have established that its cellular transport (1) is characterized by a rapid association with the target HIV cells; (2) is markedly temperature sensitive and (3) is linear over the in vitro therapeutic range. Future directions of this project include examination of similar parameters with a compound structurally similar to the above but with a 10-fold higher therapeutic index. Similar approaches are being extended as appropriate to other priority compounds.

Investigation into the Biochemical Mechanism(s) of Selective Cytotoxicity of New Antitumor Drugs

We are exploring possible mechanism(s) responsible for the selective in vitro

cytotoxicity of several ellipticine compounds toward central nervous system (CNS) tumors. Current efforts are designed to examine transport kinetics, intracellular binding and metabolism of selectively cytotoxic concentrations of drug: additional studies will examine potential strategies designed to modulate cytotoxicity of these "pro-alkylating" agents by nucleophile depletion. Similar studies are being designed and applied, as appropriate, to other priority compounds.

Biochemical Modulation of Antitumor and Anti-HIV Drugs In Vitro and In Vivo

Increased cellular accumulation of anti-HIV oxathiin derivatives has ben achieved by using a series of classical multiple drug resistance (MDR) chemosensitizers. Conversion of the active compounds to inactive intermediates in vitro by esterase has been recently demonstrated; current work is directed toward characterization of substrate specificity of these compounds for esterase, and the investigation, in vitro and in vivo, of specific inhibitory regimens designed to block detoxification. Biochemical modulation strategies are being developed and investigated with other priority compounds as appropriate.

Histological, Ultrastructural and Immunocytochemical Characterization of Human Tumor Cell Lines for Potential Use in the NCI Disease-Oriented Antitumor Drug Screen

Detailed characterizations of human tumor cell lines have been conducted to determine the suitability of individual cell lines for inclusion in the in vitro screen. Characterizations have included histological and ultrastructural observations and immunocytochemical testing with panels of antibodies directed against tissue and tumor specific antigens, and antigens associated with specific biological properties (multiple drug resistance, oncogene encoded products, etc.). The influences of the growth environment (in vitro monolayer or three-dimensional, in vivo xenograft) and other cultivation parameters on expression of cell line characteristics are also being studied.

In Vivo Model Development for Preclinical Pharmacological and Toxicological Evaluations of High Priority Compounds

Appropriate animal models are being developed for initial in vivo pharmacological and toxicological evaluations of prototype compounds discovered in the primary in vitro anticancer and anti-AIDS drug screens. Development involves comparative studies of different animal species, vehicles and routes of administration to determine optimal model parameters for the specific drug. In addition, in collaboration with other LDDRD investigators, innovative in vivo techniques for the assessment of anticancer or anti-AIDS activity of compounds are being investigated.

Structure-Activity Optimization Strategies Based Upon Pharmacokinetics, Metabolism and/or Modes of Action of Priority Compounds

Chemical structural modifications of priority compounds are being directed by pharmacokinetic and metabolic principles in order to afford structural analogs or congeners with greater in vivo efficacy than the lead compounds identified by the screens. In addition, we are investigating whether chemical structural

characterization of the principal metabolites of a priority compound may provide insight into potential mechanisms of action and a basis for the development of a drug which is more effective in vivo.

Development of Molecular Approaches to Qualitative and Quantitative Assay of Human Versus Non-Human Host Tissues: Potential Applications to Experimental Therapeutic Model Development.

We are exploring the application of molecular biologic approaches to development of new in vivo metastatic, orthotopic and other xenograft models where more precise quantitative determination of tumor burden is critical. Utilizing probes for repetitive human DNA sequences, we have demonstrated that human tumor DNA can be detected in DNA extracted from whole mouse organs and that this signal can be utilized to characterize the time-course and tissue distribution of metastatic cells. Current work is directed towards improving methods of dot-blot quantitation, application of the polymerase chain reaction to enhancement of sensitivity for detection of human DNA sequences and application of DNA probe technology to evaluation of therapeutic effects.

Development of Chemical Characterization/Dereplication Strategies

Protocols have been developed for the preliminary chemical evaluation and characterization of extracts identified by the biological screens. For organic extracts, a solvent-solvent partitioning scheme is utilized to provide five fractions of increasing polarity.; the location of the bioactivity is used to guide the selection of initial chromatographic approach; NMR analyses of the active fractions are used to search for preliminary clues as to chemical class of the active agent(s). For aqueous extracts, a four-stage chemical characterization protocol employing small cartridge chromatography technology produces a profile of the elution of the bioactive components(s); this profile is compared to those of standards, e.g. sulfated polysaccharides, for purposes of dereplication, chemical characterization and selection of appropriate fractionation method.

Development of Preparative Separation Strategies for Complex Mixtures of High Priority Natural Products

A variety of unique approaches have been adapted to scale-up problems encountered with new natural product drug development candidates. Examples of techniques include normal phase HPLC on cyano-(taxol) and amino-(alkaloids) bonded phase columns and countercurrent chromatography (alkaloids).

Investigation of a Ling Between Anti-HIV and Phorbol Receptor Binding activities

The isolation and identification of prostratin as an anti-HIV compound and the observation of a significant number of anti-HIV active extracts from the Euphorbiaceae led us to consider a linkage of phorbol receptor binding activity with AIDS antiviral activity. his concept of linkage has been supported by observation of anti-HIV activity in the aplysiatoxins and indolactams. A very small number of plant extracts outside the Euphorbiaceae are active in the phorbol receptor binding assay and the anti-HIV screen; from these leads we have discovered anti-HIV active triterpenes and xanthophylls.

Anti-HIV Alkaloids Isolated from Buchenavia Capitata

Bioassay-guided fractionation of the leaf extracts of Buchenavia capitata via partition chromatography on silica and high-speed countercurrent chromatography has led to the isolation of several piperidine-flavonoid alkaloids with anti-HIV activity. The lead compound, O-demethyl buchenavianine, shows activity at 100 ng/ml, while other homologs isolated thus far are less active. The compounds are being thoroughly characterized by ^{1}H and ^{13}C-NMR (including HMBC and HMQC) and high-resolution mass spectrometry.

Anti-HIV Sulfated Polysaccharides from a Marine Sponge and a Tunicate

Ultrafiltration, size exclusion chromatography, and ethanol trituration have concentrated anti-HIV activity of sponge extract in a high molecular weight fraction whose ^{13}C-NMR and elemental analyses are consistent with a sulfated 6-deoxy-polysaccharide. The same approach has led to similar results with an aqueous extract of tunicate. The trituration/^{13}C-NMR analysis approach is proving to be a useful confirmation for the chemical characterization/dereplication protocol's indication of sulfated polysaccharides.

Anti-HIV Dimeric Alkaloids from Ancistrocladus sp.

The organic extract of the leaves and stems of Ancistrocladus sp. was found active in the NCI AIDS-antiviral assay. Bioassay-directed separations using centrifugal countercurrent chromatography followed by HPLC on an amino bonded-phase column resulted in the identification of a novel dimeric alkaloid. In addition to the active alkaloids, several related but inactive compounds were identified. Structure elucidation and modeling studies are nearing completion; preparative isolation methods are being developed to provide additional material for biological studies.

Lutein, a Xanthophyll with Anti-HIV Activity

The organic extract of the leaves and stems of two plants, Excoecaria guineensis and Combretum bracteatum, were active in the NCI anti-HIV assay. The extracts also gave positive results in a phorbol ester binding assay. Tandem application of these two assays to direct separations led to the identification of the dihydroxy carotenoid lutein as the major active compound in Combretum; lutein was also isolated from Excoecaria extracts. The behavior of lutein and several related xanthophylls in a battery of secondary screens is being investigated.

AIDS Antiviral Plant Diterpenes

Atisane and kaurane diterpenes have been isolated from extracts of two trees from tropical rainforests, Homalanthus acuminatus and Chrysobalanus icaco. One compound from each skeletal type was marginally active; three of the isolates are novel compounds. Some insight has been gained regarding structural features important for the bioactivity. The isolates have been used as templates for installing and testing a variety of 2D-NMR experiments.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07191-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Development of Assays for "Stage II" Evaluations of New Anti-HIV Compounds

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

PI:	James McMahon	Biologist	LDDRD NCI
Others:	Robert Gulakowski	Microbiologist	LDDRD NCI
	Robert Moran	Biologist	LDDRD NCI
	Michael Currens	Microbiologist	LDDRD NCI
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Laboratory of Molecular Virology and Carcinogenesis, Advanced BioScience, Inc., FCRDC (Dr. S. Oroszlan);
Laboratory of Tumor Cell Biology, DCE, NCI (Dr. P. Nara); Laboratory of Biochemistry, DCBDC, NCI (Dr. S. Wilson)

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Cell Biology, Biochemistry, Experimental Therapeutics Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.1	1.1	3.0

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.)*

Prototype antiviral agents are being used to develop and evaluate novel assay systems aimed at elucidating potential mechanisms of action of new compounds identified by the NCI primary anti-HIV screens. The assays define specific stages in the virus life cycle and include a diverse set of biochemical endpoints. Prioritization of new lead compounds is thus facilitated through the derivation of a wider variety of in vitro test data than available from the primary screen.

Professional Personnel (cont'd)

Others:	David Vistica	Pharmacologist	LDDRD NCI
	John Cardellina, II	Suprv. Research Chemist	LDDRD NCI
	Kirk Gustafson	Senior Staff Fellow	LDDRD NCI
	John Beutler	Cancer Expert	LDDRD NCI
	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI

Objectives

1. Development and evaluation of assay systems aimed at elucidating potential mechanisms of action of new compounds identified by the NCI primary anti-HIV screen.

2. Determination of the range of biological activity of newly identified anti-HIV agents.

3. Development of methods for definitive evaluations of in vitro anti-HIV synergy; evaluation of new anti-HIV drug leads in combination with key reference compounds.

4. Support bioassay-guided isolation, identification, and chemical and biological characterization of new anti-HIV leads from natural products.

5. Utilization of the resulting expanded in vitro test data base as a means of prioritizing new synthetic and natural product-derived lead compounds for drug development.

In order for the NCI/DTP anti-AIDS drug discovery program to function optimally by bringing the best compounds to clinical trial in the shortest possible time, as much information about the new lead anti-HIV compounds is needed as quickly as possible. Follow-up confirmatory assays as well as mechanism-oriented assays are necessary to establish the degree of uniqueness of new lead compounds and to help establish their priority as potential drug development candidates. New leads with novel mechanisms of action relative to existing clinical prototypes such as AZT, dideoxycytidine (DDC), and dideoxyinosine (DDI) are urgently needed. The treatment-limiting toxicities of these agents as well as the emergence of AZT-resistant mutant virus further compound the urgency for new active drugs with different modes of action and/or more favorable pharmacological or toxicological profiles.

There are numerous events in the replicative cycle of HIV that are potential targets for anti-viral compounds, information from a diverse set of biochemical endpoints will allow for more rational selection of new prototype lead compounds for development.

In this project, our goal is to develop and apply a wide variety of in vitro

assay systems to help elucidate potential mechanisms of action of new compounds identified by the NCI primary anti-HIV screen. A major emphasis is the development of assays which can define specific stages in the HIV life cycle. A range of HIV-sensitive cell lines of the T-lymphoid, monocytoid and non-lymphoid origins will be used as will different strains of both HIV-1 and HIV-2. Assays directed toward the effects of lead compounds on specific viral proteins and enzymes will be developed. Specific viral target sites will include reverse transcriptase, HIV protease, tat functions, CD4 expression and viral protein glycosylation, as well as others.

A diverse panel of compounds with known or proposed anti-HIV mechanisms and/or properties will be utilized to define and calibrate the proposed assay systems. As this battery of biological and biochemical endpoints becomes properly validated and operational, new lead compounds discovered by the primary anti-HIV screen will be evaluated for possible preclinical or clinical development quickly and effectively.

As an adjunct to the above, in vitro combination studies will be performed to determine whether a given agent acts in a synergistic, additive or antagonistic fashion with a given reference compound. Evidence for synergy or antagonism may help elucidate whether the new compound acts via a different mechanism than the selected reference compound, whereas evidence only of additivity may imply that the new and reference compound act by the same or a similar mechanism. Although these studies are aimed primarily at assessing the possible uniqueness of mechanism of action of new leads, there may be obvious implications for possible in vivo or clinical synergy for future follow-up.

Major Findings

Confirmatory Assays: A semiautomated multiparameter assay has been developed in our laboratory. This procedure involves an XTT assay along with a battery of other interrelated assays performed in a concurrent fashion. The assays include the determinations of cell viability, DNA content, supernatant reverse transcriptase activity, p24 core antigen production and the synthesis of infectious HIV virions which are all executed in parallel from a single well of a 96-well microtiter plate containing human host cells infected with HIV. Cellular viability is determined by a fluorescence assay using the viability probe 2′-7′-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF). BCECF is a nonfluorescent molecule which readily enters viable cells where it is hydrolyzed by cellular esterases to a fluorescent molecule. DNA content is measured with the nucleic acid specific dye, 2-diamidino-phenylindole (DAPI) which fluoresces when intercalated at A-T specific sites in chromatin.

The ability to obtain multiple assay endpoints from each well of a 96-well microtiter plate has several important advantages over single endpoint determinations. The assays yield a maximum amount of antiviral and cytotoxicity information with a minimum amount of sample and may detect active compounds that may be missed by single endpoint assays. The amount of viral synthesis taking place in each well can be normalized to the amount of cellular material present. This reduces variability and takes into account the cytotoxic or static effects test compounds could have on virus production. In addition, this assay can be

used to determine whether XTT formazan production is an adequate endpoint for further second stage assays.

The expression of HIV-derived glycoproteins on the cell membranes is an early event following cellular infection with HIV. These include gp120, a viral protein associated with the tissue selectivity and cytopathology (e.g., syncytium formation) of HIV infection. Other HIV-associated antigens (i.e., gp160, gp41, and p24) either singly or in combination, have known or suggested functions in the cytopathogenicity and transmission of HIV. To explore the possibility that lead compounds could alter the expression of HIV antigens, we developed a quantitative method for measuring HIV antigen-expression. Briefly, we have used specific anti-HIV antibodies and interactive laser cytometry to measure levels of gp160, gp41, gp120, and the viral core protein, p24, in HIV-infected cell lines. This method permits early, sensitive, quantitative 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. Preliminary studies of the effects of AZT on viral antigen expression confirmed the potential utility of this technique. Interactive laser cytometry is also used to measure intracellular calcium concentrations during HIV infections and drug treatments.

The most recent assay that has been developed and implemented in our laboratory uses a combination of culture manipulations to estimate more specifically where in the virus life cycle a compound exerts its antiviral activity. Briefly, the assay involves incubating cells with the test drug prior to and during infection and then continuing drug incubation for various times. The compound is then removed and the cells are reincubated with medium alone, test drug or antiviral compounds with known (or suspected) mechanisms of action such as DDC, dextran sulfate and srCD4. In the CEM-SS/HIV_{RF} cell system, DDC is very effective early in aborting the HIV infection. However if DDC is added several hours after infection its protective response is lost. If cells are infected in the presence of oxathiin carboxanilide (OC) and treated with this compound for several hours, removal of the drug and addition of DDC as late as 24 hours after infection protects the cells from the cytopathic effect of HIV infection. In contrast, when OC is removed and the cells placed in medium alone, there is complete cell killing for up to 24 hours. Cell killing is also evident in cultures that receive DDC alone 24 hrs after infection. Collectively these studies, using several of the secondary assays that have been developed to date, implicate a novel mechanism of action of OC involving the blockage of virus fusion to the plasma membrane.

Proposed Course

A principal goal of this project will continue to be the application of any or all of the above battery of newly-developed methodologies to the "stage II" evaluations of high-priority new anti-HIV leads identified by the NCI/DTP primary screen. In addition, we will continue to expand the scope of our stage II methodologies, both through experimental developments in our laboratory as well as through collaborations with other researchers.

Warren JT, McMahon JB, Weislow OS, Gulakowski RJ, Kiser RF, Boyd MR. Interactive laser cytometric analysis of retroviral protein expression in HIV-infected lymphocytic cell lines. AIDS Res Hum Retroviruses 1990;6:1131-37.

Bader J, McMahon JB, Schultz R, Narayanan V, Pierce JB, Harrison WA, Weislow OS, Midelfort CF, Stinson SF, Boyd MR. Oxathiin carboxanilide, a novel inhibitor of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 1990;in press.

Gulakowski R, McMahon JB, Staley PG, Moran RA, Boyd MR. A semiautomated, multiparameter assay for anti-HIV drug screening. 1990;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07192-01 DDRD

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October 1, 1990 to September 30, 1991

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

In Vitro Methods for Anticancer Drug Screening and Stage II Evaluation

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

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Others:	Susan Kenney	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI
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COOPERATING UNITS *(if any)*

Program Resources, Inc. (Dr. A. Monks, Dr. D. Scudiero)

LAB/BRANCH

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SECTION

Cell Biology, Biochemistry and Experimental Therapeutics Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	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.)*

A variety of alternative in vitro antitumor assays are being developed and comparatively evaluated with a view toward possible applications in primary (Stage I) screening or to help prioritize (Stage II) new agents for further study. Specifically, new methods are being explored for determination of cell survivorship of drug treatment; recovery of proliferative capacity following drug treatment; kinetics of drug action; pulse duration effects on cellular responses; and optimization of in vitro chemotherapeutic schedules. Moreover, methods for assessment of the above not only in monolayer culture but also in single-cell suspensions, weakly adherent lines, floating aggregates and spheroids are being pursued.

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John Beutler	Cancer Expert	LDDRD NCI
Kirk Gustafson	Senior Staff Fellow	LDDRD NCI

Objective

The objective of this project is to develop new rapid in vitro assay methods for a) screening large numbers of new compounds for selective antitumor activity (Stage I) or b) to prioritize lead compounds with interesting selectivity profiles for more detailed evaluation (Stage II).

The specific aims of this project are:

1. Development of a generally applicable in vitro assay method for estimating the growth and drug response of single cell suspensions, adherent and weakly adherent monolayers, and floating cell aggregates.

2. Development of in vitro Stage II methods to help select, prioritize, and determine optimal in vitro chemotherapeutic scheduling of new lead compounds.

3. Collaboration, as appropriate, to compare/contrast and evaluate results of other Stage II evaluation strategies of priority agents.

4. Collaboration within LDDRD as appropriate to support bioassay-guided isolation, identification, and chemical and biological characterization of new antitumor leads from natural products.

Methods

Cell Culture: Human tumor cells are grown in RPMI 1640 with glutamine and 5% fetal calf serum. Cells are dissociated with 0.1% trypsin plus 3 mM CDTA in NKT buffer (145 mM NaCl, 5.4 mM KCl, 10 mM tris, pH 7.4). Stocks are split weekly and maintained in T75 or T150 flasks with 50 and 100 ml respectively of growth medium changed three times weekly. Seeding densities are individually determined for each cell line. A number of the experiments described below use the Stage I in vitro format. Cells are seeded in 96-well plates at moderate seeding densities (most commonly 5-20 thousand cells/well) selected individually for each cell line on the basis of doubling time. They are preincubated for 1 day to allow recovery from dissociation, incubated continuously for 2 days, then harvested for SRB cell protein assay.

SRB Assay: Unless otherwise noted, growth and cytotoxicity are measured by the sulforhodamine B method developed in this laboratory. Briefly, cells are fixed in situ for 30 minutes by layering one volume of 50% TCA on top of 4 volumes of growth medium overlying the cell culture. The fixed cultures are water washed

4 times, stained with 0.4% SRB in 1% acetic acid for 30 minutes, washed 5 times with 1% acetic acid, then air dried. SRB is solubilized with 10 mM unbuffered Tris base and optical density measured spectrophotometrically at 520 nm with a Molecular Devices UVmax 96-well plate reader.

Propidium Iodide Assay: A cuvette assay was developed for sample volumes of 1 ml or more. To each ml of growth medium was added 0.125 ml of a cocktail containing 2.5% N-laurylsorcosine (NLS) and 200 μg/ml of PI. Samples were lysed (NLS) for 1 hour at room temperature, mixed well with a pasteur pipet, allowed to stand for 5 minutes to clear air bubbles, then read fluormetrically at excitation/emission wavelengths of 530/605 nm. Resolution was comparable to the SRB assay. An optimized protocol was developed for subconfluent and multilayered cultures using protocols similar to those which we employed in developing the naphthol yellow S (NYS) and SRB assays. Dose-response and kinetic experiments were used to determine appropriate supramaximal dye concentrations and incubation times. Similar experiments were performed to determine the optimal detergent concentration and sample digestion time.

Kinetics of Drug Action: Replicate cultures were continuously incubated with test compounds at multiple concentrations for 1, 2, 5, 7, 24, 48, 72, and 96 hours to determine the kinetics with which cellular response develops as measured by the SRB assay. A continued dose-response development beyond 2 days indicates a better potency or efficacy than was reflected by Stage 1. A very rapid dose-response development will eliminate the need for detailed pulse duration studies, which have higher contamination rates because of the more extensive handling which they require. Initially, kinetic studies were performed on subconfluent cultures using the Stage I culture protocol. However, because culture architecture often influences drug potency and efficacy, the experiment was repeated on focal multilayered colonies. This permits the comparison of a drug's effectiveness against rapidly growing subconfluent and slowly growing 3-dimensional cultures.

Pulse-Duration Studies: As a default protocol, replicated cultures will be pulsed for 1, 2, 5, 7, and 24 hours with multiple drug concentrations. At the end of the pulse, test solution were removed and replaced with fresh growth medium. The cultures were incubated until the 48th hour, then collected along with cultures exposed continuously to the drug for a full 48 hours. Where appropriate, this protocol was modified on the basis of results obtained from the kinetics studies of the previous section. As with the kinetic studies, pulse-duration experiments were performed first on subconfluent cultures using the Stage I protocol, then on focal multilayered cultures.

Cell Survivorship: Immediate survivorship was determined by dissociating cells at the end of a drug pulse and replating them in a T25 flask. The duration of the pulse was a supramaximal period determined from the preceding pulse-duration study. Following an overnight incubation period for cell adhesion, the number of adherent cells with one or more visibly extended processes (lamellae, filopodia, pseudopods, neurites) per unit area were determined for both test and control populations. Adhesion and process extension are both active processes which depend upon the functionality of a variety of metabolic pathways and intracellular organelles. This adhesive survival assay provides an estimate of cells metabolically viable at the end of drug insult. A replicate set of

cultures was assayed for colony forming efficiency (CFE). Following drug treatment, cells were dissociated and plated out at 500 cells per T25 flask. Cultures were retained for one to two weeks, depending on doubling time, then scored for CFE. The frequency distribution of colony sizes was determined for test and control samples. Differences in the mode, dispersion, and skew of the distributions are used to determine whether a drug has changed CFE. This provides a preliminary estimate of proliferative viability. Many but not all of the lines in the current screen meet this requirement. CFE was determined for both subconfluent and focal multilayers.

Proliferative Recovery: Focal multilayered colonies (FMCs) were used to evaluate proliferative recovery following drug treatment. Colonies were preincubated for one week to allow multilayering, exposed to a drug for a supramaxial period of time determined from pulse-duration studies, washed, and incubated in drug-free medium for 4 weeks or until obvious proliferative recovery has occurred. Samples were collected at time zero, end of drug pulse, and on days 7, 14, 21, and 28. To minimize the loss of samples to contamination, there was no feeding during the recovery period. Seeding conditions are selected so that control FMCs are in growth or plateau but not in decline phase on day 21. For rapidly growing lines, this is the limit beyond which control cells begin to die out from acute nutrient deprivation. With slowly growing FMCs and with focal subconfluent colonies (FSCs), controls can usually be carried an additional week or more. Test cultures which have experienced regression or growth inhibition can often be maintained for several additional weeks, extending the ability of the assay to evaluate growth delay and recovery.

Schedule Optimization: FMC colonies were used to explore the effectiveness of different regimes. Cultures were treated with from 1 to 5 courses of chemotherapy using optimal concentrations and pulse-durations determined from preceding Stage II assays. Intervals between courses depend on pulse-duration, but generally range from 1 to 10 days initially. Where appropriate, these regimes are revised in the light of initial results. Because each round of treatment involves a change of growth medium, these experiments can be continued for a number of weeks.

Major Findings

Stage I Assays: The SRB assay currently used in the NCI's primary Stage I in vitro drug screen was originally designed for use with substratum-attached cells. Later, panels for leukemia and small cell carcinoma of the lung (LSC) were added to the screen. The leukemias grow as single cell suspensions, while most of the LSC lines grow as a mixture of attached cells and floating multicellular aggregates. We found that single cell suspensions and very small aggregates could be cross-linked to the substratum by gently layering 80% trichloroacetic acid (TCA) on top of the culture growth medium provided cells were physically in contact with the plastic at the time of fixation. This procedure requires careful handling of samples, and is susceptible to movement artifacts. While the SRB assay is adequate for single cell suspensions when used carefully, it is not ideal. The assay does not work at all with aggregates containing more than 4-5 cells, a limitation that excludes a number of cell lines from use in the screen. We are therefore attempting to develop for possible use in Stage

I screening, a new colorimetric cytotoxicity assay that does not require fixation or washing steps that might cause cell loss artifacts.

Stage II Assays: Stage II in vitro assays are intended to provide additional information needed to help prioritize compounds for further in vitro and in vivo investigation. They address the questions of how quickly a drug achieves its effectiveness, how brief an exposure period this requires, how many cells survive treatment, whether there is a proliferative recovery following treatment, and if so when this recovery begins. The Stage II assays may also help to identify artifacts that cannot be resolved in the Stage I assay.

New Potential Stage I Cytotoxicity Assay: Although a large number of colorimetric assays have been reported in the literature, few permit the quantitation of cellular material without either fixation, washing, or sample transfer. One exception is the propidium iodide/india ink method of van Lambalgen and Lelieveld. Although this assay was unsuitable for our purposes, it provided a starting point for the development of an alternate Stage I assay potentially useful for single cell suspensions, adherent and poorly adherent monolayers, and floating cell aggregates. Propidium iodide (PI) and ethidium bromide (EB) exhibit a 20-30 fold fluorescence enhancement when bound to double stranded regions of RNA and DNA. An attendant shift in their absorption spectra permits a total enhancement of up to 100-fold with carefully selected excitation and emission wavelengths. For these reasons both dyes have found widespread use as quantitative stains for RNA and DNA. These dyes are not specific for polynucleic acids, however. They electrostatically bind to negative counter ions, including those of proteins and glycosaminoglycans. This is a disadvantage with samples that have high background levels of an interfering substance such as the serum protein of growth medium. We have identified an excitation/emission wavelength combination that allows a propidium iodide signal to be read fluorometrically without interference from fetal calf serum at concentrations of up to 10%. In cuvette measurements of cell numbers using these wavelengths, PI is as sensitive an assay of cell number as SRB. A 96-well fluorescence plate reader has recently been acquired. When the appropriate excitation and emission filters are received, we will attempt to extend the PI assay to a 96-well plate format.

Stage II Assays: One of the first study sets committed to Stage II in vitro evaluation were the cytochalasins. Cytochalasins A-H were initially tested in the NCI Stage I anticancer screen at five, 10-fold dilutions starting at 100 μM. All exhibited selective activity against the renal and CNS panels. In addition, cytochalasin A (CCA) exhibited weak anti-leukemic activity. For stage II analyses, the effects of cytochalasins A-J were examined in detail using the RXF-393 and A-498 renal lines as drug-sensitive in vitro models. All of the cytochalasins caused a rapid rounding up of cells. At 10 μM, this process began within a time period ranging from a few tens of seconds to a few minutes. Lamellae, filopodia, pseudopodia, and neurites were largely retracted leaving a cell body that was quasi-spherical or ellipsoidal. Over a time scale of a few minutes to a few tens of minutes, cytoplasmic blebbing became visible at higher concentrations. Small balloon-like projections of cytoplasm extended out from the cell body. In some cases an asymmetric cytokinesis occurred: blebs pinched off from the cell, becoming free-floating minicells devoid of nuclei. Blebbing is a prominent feature of the apoptosis cell death cascade that can be initiated

by a variety of different stimuli. There is evidence in other systems that Ca influx may be the excitation-response coupling mechanism that triggers the cascade. Cellular rounding greatly reduces the surface contact area between cells and substratum, causing a considerable loss of adhesiveness. Routine handling operations, such as the aspiration or addition of fluids, cause local cell detachment. If the detached cells fail to readhere before TCA fixation, there is an artifactual loss of cellular material. This is likely to be a common occurrence in the stage I assay, and is a documented complication in the stage II. The possibility, therefore, had to be considered that the apparent selective cytotoxicity of the cytochalasins represented a differential anti-adhesive effect rather than differential growth inhibition or cell killing. There was relatively little overt lysis by cytochalasin treated cells, particularly in the first 2-3 days of treatment. Although occasional cells were seen in the terminal lytic stage of colloid osmotic swelling, most cells remained morphologically intact. A small proportion of cells became highly crennulated with a sharply reduced cytoplasmic volume, a characteristic of apoptosis. The kinetics of drug action against RXF-393 cells were examined over a several day period with continuous drug incubation. Cytochalasin D was tested at 1-100 μM. The remaining drugs were tested at 0.1-10 μM. Net growth inhibition was not detectable within the first 8 hrs of incubation, but was generally apparent at 24 hrs. Net cell killing took 2-3 days to develop. Cytochalasin E was the most potent of the cytochalasins. Cytochalasins D, H and J were reasonably potent as well. With most of the drugs, stronger cytotoxicity was observed with 3 and 4 days of incubation than with 2 days. However, starvation artifacts became evident in control cultures at between 48 and 72 hrs. It is possible that the apparent increase in cytotoxicity at later times reflects nutritional deprivation rather than a drug effect. Cells were drug-pulsed for 0, 2, 4, 6, 8, and 24 hrs then incubated in drug-free medium until hr 48. Higher concentrations were usually near-maximally or maximally effective with 2 hrs pulses and 48 hrs of continuous incubation. Adhesion and spreading are active events which require the functional integrity of a variety of intracellular structures, organelles, and processes. A-498 cells treated with control or test solutions for 48 hrs were dissociated and transferred to 8-well plates. Following overnight incubation, the number per unit area of adherent cells with one or more visibly extended processes was determined. This served as a measure of short term survival following toxic insult. With both cytochalasins E and D, short term survivorship was 10-15% of the control populations. Since the drug treated samples had regressed, while the control population had expanded, the proportion of treated cells which survived was higher than the 10-15% figure. The ability of individual cells to serve as founders of progressively growing colonies provides a longer term estimate of reproductive survivorship. The frequency of colonies with 10 or more cells 1 week after a 48 hr CCE or CCD treatment was used as a CFE estimator for A-498 cells. By this criterion, there was zero colony formation at 1 μM cytochalasin E. However, in the unit area scored, there were 325 cells surviving either as singlets or small colonies (< 10 cells) at 1 μM, and 65 at 10 μM. Some of these were clearly proliferative, albeit more slowly growing than cells which gave rise to larger colonies. With cytochalasin D, there was nearly 50% survivorship by the CFE criterion even at 10 μM. In long-term focal multilayer recovery assays with RXF-393 cells, all of the cytochalasins showed recovery and progressive growth following a 2 hr pulse with 1 μM of drug. Cytochalasin E exhibited no recovery for 5 weeks following a 10 μM pulse.

It was concluded that while several of the cytochalasins could cause partial regression and significant growth delay, none could eradicate a target population of sensitive cells; maximum regression was limited to one log, and target populations recovered following drug removal.

Proposed Course

This project will be continued under contract.

Publications

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107-12.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, Boyd MR. Feasibility of a high-flux anticancer drug screen utilizing a diverse panel of human tumor cell lines in culture. J Natl Cancer Inst 1991;83:757-66.

Skehan P. A microenvironmental model of mammalian cell cycle variability. In WR Paukovits (ed.), Growth Regulation and Carcinogenesis, Vol. II, CRC Press, Boca Raton, 1991, pp 313-25.

Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR. Tetrazolium based assays for cellular viability: A critical examination of selected parameters affecting formazan production. Cancer Res 1991;51:2515-20.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07193-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Development and Application of Agar-Based Stage II Antitumor Assays

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

PI:	Michael Alley	Pharmacologist	LDDRD NCI
Others:	Christine Pacula-Cox	Biologist	LDDRD NCI
	Tanya Prather	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI
	Robert Shoemaker	Biologist	LDDRD NCI
	Philip Skehan	Cancer Expert	LDDRD NCI

COOPERATING UNITS *(if any)*

Information Technology Branch, DTP (Dr. Paull); Biometrics Research Branch, CTEP (Dr. Rubinstein); Program Resources, Inc., FCRDC (Dr. Monks, Dr. Scudiero)

LAB/BRANCH

Laboratory of Drug Discovery Research and Development

SECTION

Cell Biology, Biochemistry and Experimental Therapeutics Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	1.2	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.)*

Conventional agar-based assay systems have been adapted (a) to evaluate agents exhibiting differential in vitro test results in the Stage I cancer screen, (b) to identify and quantitate cell populations which may survive such treatment, and (c) to compare profiles of cell line drug sensitivity observed in Stage I and Stage II assays. Also, the development of an under-agar microculture tetrazolium assay system appears well-suited for accurate drug sensitivity determinations of compounds which liberate cells from fluid monolayer culture systems (i.e., false-positive test results due to chemical and/or physical properties of the test material). Such a culture format is amenable not only to conventional colorimetric analysis but also to image analysis which affords a far greater detection range (4-5 logs) and which does not require the use of organic solvents. In addition, we are developing methods to support new in vivo/in vitro drug assay strategies based upon the culture of pre-formed colonies and xenograft-cultivated material (coupled with ex vivo bioassays) which should help provide experimental insights concerning in vivo drug efficacy (or lack thereof). Overall, the agar-based assay protocols are designed (a) to identify agents which appear to have the greatest potential for in vivo efficacy and (b) to eliminate agents lacking appropriate pharmacologic profiles, and (c) to permit isolation and subsequent characterization of drug-sensitive and -insensitive cell populations within screening panel cell lines.

Personnel (cont'd)

Others:	David Vistica	Pharmacologist	LDDRD NCI
	John Cardellina II	Suprv. Research Chemist	LDDRD NCI
	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI
	John Beutler	Cancer Expert	LDDRD NCI
	Kirk Gustafson	Senior Staff Fellow	LDDRD NCI

Objectives

1. Investigation of whether differential drug activity observed for selected priority agents in the Stage I cancer screening assays occurs in agar-based in vitro assay systems.

2. Identification of agents which may have given "false-positive" Stage I screening results due to physical or chemical drug perturbations in fluid cultures.

3. Development of new agar-based in vitro evaluation systems (a) which may more closely approximate solid tumor nodules found in patients, and (b) in which drug exposure may more closely approximate in vivo conditions; or which may offer distinct, technical advantages over current, single cell assay methodologies.

4. Performance of pharmacologic calibration studies and the development of specialized in vivo/in vitro drug evaluation strategies.

5. Collaboration, as appropriate, to compare/contrast and evaluate results of other Stage II evaluation strategies of priority agents.

6. Collaboration within LDDRD as appropriate to support bioassay-guided isolation, identification, as well as chemical and biological characterization of new antitumor leads from natural products.

Methods

We are investigating the suitability of soft agar assay systems for Stage II in vitro drug evaluations for several reasons: (a) these methodologies represent an alternate culture format which has been widely used by cancer research investigators for experimental drug evaluations as well as radiotherapeutics evaluation for many years; (b) cultures are amenable to automated analysis as well as to subsequent close scrutiny by microscope and observer interpretation; (c) specialized culture protocols can be designed to address several types of questions relevant to experimental drug evaluation; and (d) soft agar cultures provide a useful means to isolate specific tumor cell line subpopulations from cell lines as well as xenografts for detailed histologic and quantitative morphologic evaluations.

Protocols employed in the NCI/DTP Stage I antitumor screen have several conceptual and operational limitations which need to be addressed in subsequent steps of experimental drug evaluation. In particular, Stage I in vitro procedures have certain limitations which can be corrected or interpreted more

clearly based upon specialized Stage II assay systems: (a) difficulty in the cultivation and or analysis of non-adherent cell lines (especially leukemia/lymphoma and small cell lung carcinoma); (b) potentially erratic results for certain cell lines which exhibit slow recovery in 48-hour drug exposure assays; and (c) detection of apparent cell kill due to adherent cell detachment rather than drug-induced cytotoxicity. In addition, questions have arisen concerning physical perturbations imposed by insoluble test agents upon the survival of cells which require attachment to coated plastic culture vessels for growth. Moreover, from the standpoint of selecting priority agents from the Stage I screen it may be important to know whether selected agents exhibit "differential" effect among cell lines are due to selective sensitivity, or alternatively, due to the presence of differentially "resistant" tumor cell subpopulations in some of the human tumor cell line cultures.

Therefore, in this project, the evaluation of selected compounds in Stage II in vitro assays is aimed at (a) providing comparative quantitative data for the same cell lines employed in the Stage I screen (i.e., both dose-response curves and multiple drug-effect indices), (b) eliminating in vitro "false-positive" agents and the prioritization of new agents for in vivo investigation, and (c) permitting interpretations of the basis for differential effect amongst drug-sensitive and drug-insensitive cell lines. In addition, the soft-agar methodologies provide relevant culture material for comparative morphologic/biologic evaluations of selected priority agents in collaboration with other LDDRD investigators.

The development and applications of soft agar culture procedures have also been prompted by (a) certain observations from in vitro and in vivo screening of priority agents (e.g., cytochalasin-induced cell detachment and the presence of drug-insensitive subpopulations in screening panel cell lines), (b) anticipated problems associated with testing insoluble constituents derived from natural products, and 3) the need to develop in vivo/in vitro test strategies which can help to identify/expedite priority agents appearing to have the greatest potential for in vivo efficacy and which can quickly eliminate agents lacking appropriate pharmacologic profiles.

Major Findings

Initially, our experimentation with the standard soft agar colony formation assay (SACFA) focused upon two practical issues: (a) whether the method is applicable to human tumor cell lines employed in the DTP drug screening program; and (b) whether the methods of culture analysis and data processing could be sufficiently automated to permit Stage II drug evaluations on a moderate scale. In addition, we have made technical improvements and conceptual modifications in the standard SACFA: (a) development of a colorimetric analysis procedure for initial determinations of effective drug concentration range and the analysis of cultures exhibiting little biological activity or questionable differential activity amongst cell lines, and (b) development of a soft agar based assay containing pre-formed colonies at the time of drug application to serve as a more stringent in vitro test of agents exhibiting lethal activity in single cell cultures. We have utilized both standard and specialized SACFA to evaluate selected priority agents identified in the Stage I cancer screen.

Specialized microcomputer software for all agar-based assays has been developed for the immediate collection, analysis, interpretation and database tabulation

of test results. The same sets of data (ASCII format) are amenable to direct uploading to mainframe computers for purposes of statistical analysis and comparisons of data for the same cell lines generated by others in our laboratory and other groups associated with the Developmental Therapeutics Program.

Proposed Course

This project is being terminated.

Publications

Alley MC, Hursey ML, Pacula-Cox CM, Rubinstein L, Boyd MR. Morphometric and colorimetric analyses of human tumor cell lines growth and drug sensitivity in soft agar culture. Cancer Res. 1991:51;1247-56.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07194-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

In Vitro Cellular Pharmacology of New Anti-HIV and Antitumor Drugs

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

PI:	David Vistica	Pharmacologist	LDDRD NCI
Others:	Miriam Hursey	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI
	Robert Shoemaker	Biologist	LDDRD NCI
	Louis Malspeis	Suprv. Research Chemist	LDDRD NCI
	James McMahon	Biologist	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Cell Biology, Biochemistry and Experimental Therapeutics Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

This project currently is directed toward the elucidation of mechanism(s) of drug transport, intracellular binding, distribution and detoxification. Current studies of a novel oxathiin carboxanilide (NSC 615985) identified by the primary NCI anti-HIV screen have established that its cellular transport (1) is characterized by a rapid association with the target HIV cells; (2) is markedly temperature sensitive and (3) is linear over the in vitro therapeutic range. An additional objective of this project is to increase drug efficacy by modulation of drug transport and detoxification. Increased cellular accumulation of oxathiin carboxanilide has been achieved by using a series of classical multiple drug resistance (MDR) chemosensitizers. Conversion of the active compounds to inactive intermediates in vitro by esterase has been recently demonstrated; future work will be directed toward characterization of substrate specificity of these compounds for esterase, and the investigation, in vitro and in vivo, of specific inhibitory regimens designed to block detoxification.

Objectives

1. Investigation into the mode(s) of cellular transport of new, high-priority anti-HIV and antitumor agents identified by the NCI primary screens.

2. Investigation into the role of multiple drug resistance (MDR) chemosensitizers as modulators of cellular transport of NSC 615985.

3. Development of strategies for inhibition of esterase mediated degradation of NSC 615985 and NSC 629243.

4. Identification of esterase-resistant analogs of oxathiin carboxanilide (NSC 615985) and an analog, NSC 629243.

Methods

Growth of CEM-SS Cells: The HIV target CEM-SS cells are grown in suspension utilizing sterile culture conditions in antibiotic-free RPMI 1640 medium containing 5% fetal bovine serum. Cells are subcultured twice weekly when they attain mid-logarithmic phase of growth (8-10 x 10^5 cells/ml).

Cellular Transport of NSC 615985 by CEM-SS Cells: Briefly, cells are harvested from growth medium by centrifugation for 5 minutes at 300 x g, washed twice in fresh growth medium and resuspended at a cell concentration of 1.25 x 10^6 cells/ml. Cellular uptake is terminated by centrifugation of a 400 μl aliquot of cells in a Millipore 0.45 micron cellulose acetate filter unit at 300 x g for 15 seconds. The filters containing cells are removed, solubilized in 0.2N NaOH and counted by liquid scintillation spectrometry. Cell-free drug blanks are run in parallel to allow correction for binding of the drug to the filter.

In Vitro Cellular Metabolism of NSC 615985 and NSC 629243: CEM-SS cells are exposed to 1 μM of each drug for 24 hrs at 37°C in RPMI 1640 medium containing 5% fetal bovine serum. Cells are centrifuged at 300 x g, extracted with methanol and aliquots examined by thin layer chromatography on Silica gel in either methylene chloride:ethyl acetate (9:1) or n-butanol:acetic acid:H_2O (4:1:1). These solvent systems effectively separate parent drug (R_f = 0.8) from polar metabolites (R_f = 0.1).

Quantitation of B-esterase (E.C. 3.1.1.1) Activity: Esterase activity is quantitated using p-nitrophenol acetate as a substrate and monitoring the absorbance of p-nitrophenol at 405 nm. Specific activity of the enzyme is determined using the method of Bradford for quantitation of protein.

Esterase-Mediated Catalysis of NSC 629243: Two methods were investigated for monitoring, in vitro, esterase degradation of NSC 629243. Preliminary experiments indicate that it may be feasible to utilize the shift in the absorbance spectrum which occurs following catalysis and/or the appearance of a product peak corresponding to isopropyl alcohol. Additionally, the thin-layer chromatographic system described above was evaluated with [^{14}C] NSC 629243 and utilized to separate parent drug from catalytic products.

Quantitation of Inhibition of Esterase-Mediated Degradation of p-Nitrophenyl Acetate and NSC 629243 by bis (p-Nitrophenyl) Phosphate In Vitro: Inhibition of esterase-catalyzed formation of p-nitrophenol from p-nitrophenyl acetate was quantitated at 405 nm. Similar inhibition of NSC 629243 degradation by esterase was monitored, as described above, either spectrophotometrically or with radiolabeled drug utilizing thin layer chromatographic separation of parent drug from metabolites on silica gel in a solvent system consisting of methylene chloride: ethyl acetate (9:1). Parent drug (R_f = 0.8) and esterase metabolites (R_f = 0.1) are easily separated in this system.

Esterase-Sensitivity of Analogs of NSC 615985 and NSC 629243: Analogs of NSC 615985 and NSC 629243 were examined in vitro for susceptibility to metabolism by porcine liver esterase using either thin layer chromatography on silica gel (n-butanol:acetic acid:water 4/1/1) or HPLC to separate the more polar metabolites from parent drug. Shifts in absorption spectra and quantitation of metabolite formation spectrophotometrically, as outlined above, were examined.

Major Findings

Time Course for Association of NSC 615985 with CEM-SS Cells: The results of these studies indicates that association of the drug with the CEM-SS cells occurs very rapidly (< 30 seconds). This initial cellular event is followed by a second phase in which there is a progressive increase in cell-associated drug which reaches maximum levels 10-15 minutes following drug exposure. These studies suggest that the drug is internalized, as opposed to being exclusively membrane bound, since cell-associated radioactivity far exceeds the inulin space, an extracellular space indicator for these cells. Uptake of NSC 615985 is exquisitely temperature sensitive. A 5-6 fold reduction in cell-associated drug occurs when cells are exposed to the drug at 4°C.

NSC 615985 Concentration and Cellular Uptake by CEM-SS Cells: The association of NSC 615985 with the CEM-SS cells is linear over the therapeutic range of 0.1 to 1.0 μM. No evidence of saturability was found. Analysis of the data using a linear, non-inverted kinetic plot indicates that a constant ratio of NSC 615985 concentration:initial velocity is maintained with increasing drug concentration. This suggests that, in the in vitro therapeutic concentration range, the transport of NSC 615985 occurs by diffusion.

Efflux of NSC 615985 from CEM-SS Cells: Exposure of CEM-SS cells to therapeutic concentrations of the drug for time periods varying from 30 minutes to 24 hrs followed by resuspension in drug-free medium results in a rapid loss of drug from the cells resulting in subtherapeutic concentrations. These results suggest that the drug is not firmly bound to its cellular target and indicate the need to maintain constant extracellular concentrations either by utilization of a sustained release mode of drug administration or implementation of a strategy designed to promote cellular retention.

Lack of Cellular Metabolism of NSC 615985 by CEM-SS Cells: Studies were initiated to determine whether cellular metabolism of NSC 615985 occurs by the CEM-SS cells. Exposure of cells for 24 hrs to the drug followed by methanol extraction and thin layer chromatography indicates that the drug is not substantially metabolized by these HIV-target cells. Approximately 94% of cell-

associated label migrates with an R_F of 0.61, a value identical with that of parent drug.

Effect of MDR Chemosensitizers on NSC 615985 Transport: Increased cellular accumulation of NSC 615985 by the HIV-target CEM-SS cells has been achieved using classical multiple drug resistant (MDR) chemosensitizers such as verapamil and quinidine.

Effect of Esterase on the Biological Activity of NSC 615985 and 629243: NSC 615985 and NSC 629243, a structurally related analog, are excellent substrates for porcine liver esterase. Exposure of these anti-HIV compounds, in vitro, to the enzyme results in a loss of antiviral activity. The esterase inhibitor bis(p-nitrophenyl)phosphate (BNPP) completely blocks esterase mediated degradation of NSC 629243 resulting in full retention of biological activity.

Proposed Course

Inhibition of Carboxylesterase Mediated Cleavage of NSC 629243: The observed in vivo degradation of NSC 615985 and NSC 629243 and the in vitro conversion of both drugs to inactive polar metabolites by porcine liver esterase is the basis for future work designed to inhibit in vivo metabolism of NSC 629243. The latter compound will be utilized exclusively for these studies since its therapeutic potency is 10-fold greater than NSC 615985 and, therefore, appears to be the better candidate for development to clinical trial.

Initially these studies will address, in vitro, several critical areas relating to measurement of esterase activity and development of an assay for quantitation of esterase mediated cleavage of NSC 629243. The latter studies will provide pertinent information relating substrate affinity (Km) of the drug for esterase to therapeutically effective concentrations. These studies will, in turn, serve as the basis for the design of detailed in vitro experiments which will investigate the ability of selected known inhibitors of esterase, e.g., bis(p-nitrophenyl) phosphate to inhibit the conversion of NSC 629243 to therapeutically inactive compounds.

Identification of "Esterase-Resistant" Analogs of NSC 629243: A considerable body of information exists concerning the catalytic properties of the carboxylesterases. The recent identification of several additional structural analogs of NSC 615985 and NSC 629243 which exhibited substantial anti-HIV activity in the antiviral drug screen provides the opportunity to utilize these observations to select analogs and to examine, in vitro, their susceptibilty to cleavage by esterase. New structures which retain the anti-HIV activity but which are resistant to cleavage by esterase will be sought.

The sensitivity of analogs of NSC 615985 and NSC 629243 to metabolism by esterase will be investigated in vitro using porcine liver esterase. Porcine liver esterase is similar to human liver esterase with regards to molecular weight, subunit weight, amino acid composition, inhibition by bis (p-nitrophenyl) phosphate and substrate specificity. The proposed studies will involve: (1) monitoring the susceptibility of each analog to metabolism by porcine liver esterase by both thin layer chromatographic and HPLC separation of the polar metabolites; (2) bioassay of esterase treated analog to determine relative anti-

HIV efficacy; and (3) the ability of bis(p-nitrophenyl) phosphate to inhibit analog breakdown.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07195-01 DDRD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical Mechanism(s) of Selective Cytotoxicity

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

PI:	David Vistica	Pharmacologist	LDDRD NCI
Others:	Miriam Hursey	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI
	Michael Alley	Pharmacologist	LDDRD NCI
	Philip Skehan	Cancer Expert	LDDRD NCI
	Sherman Stinson	Pathologist	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION
Cell Biology, Biochemistry and Experimental Therapeutics Section

INSTITUTE AND LOCATION
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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 research effort of this project has concentrated on detailed investigations into the observed selectivity, in vitro, of several quaternized ellipticine derivatives for central nervous system (CNS) cell lines. NSC 627505, a water soluble analog, is taken up 3-12 fold more by responsive CNS tumor cell lines (U-251 glioblastoma; SNB-75 astrocytoma) than by selected refractory non-CNS cell lines (HCT-15 colon adenocarcinoma; A-498 renal cell carcinoma). The observed difference in uptake between CNS and non-CNS cell lines increases with time, suggesting the participation of a carrier-mediated drug transport process in the CNS tumor cell lines.

Objective

Investigations of the mechanism(s) of selectivity of new antitumor drugs; currently this new project is focusing efforts on NSC 627505 (9-methoxy-2-methylellipticinium acetate), an ellipticine analog which exhibited selective cytotoxicity toward human brain tumor cell lines in the NCI/DTP stage I screen (Figure 1, Table 1).

Methods

1. Growth of tumor cell lines: The CNS tumor cell lines (U-251 and SNB-75) and the non-CNS lines (HCT-15 and A-498) are grown in antibiotic-free RPMI 1640 medium containing 5% fetal bovine serum. Cultures are maintained in 75 cm^2 culture flasks in a humidified atmosphere of 95% air, 5% carbon dioxide and are passaged at 85-90% of confluence.

2. Cellular transport of NSC 627505 by U-251, SNB-75, HCT-15 and A-498 human tumor cell lines: Cells in monolayer culture are rinsed with 5 ml of transport medium composed of Dulbecco's PBS containing 0.1 mM bovine serum albumin and 0.25% D-glucose. Cultures are then exposed to radiolabelled drug for an appropriate time. Radiolabelled drug is removed, plates placed on ice and the cultures rinsed three times with 5 ml, 4°C PBS. Cell protein is solubilized overnight in 1 ml of 0.2 N NaOH and aliquots utilized for both determination of cell protein and radioactivity.

Major Findings

1. NSC 627505, a quaternary ellipticine analog, exhibited selective growth inhibition and cytotoxicity toward human brain tumor lines relative to most other lines in the panel.

2. Sensitive CNS tumor cell lines accumulate 3-12 fold more than resistant non-CNS cell lines.

3. The differential sensitivity of CNS cell lines to the drug is related to the length of drug exposure.

Proposed Course

1. The observation that differential sensitivity of the CNS tumor cells is related to increased cellular uptake will serve as a basis for detailed investigations designed to characterize the transport system utilized by the drug. These investigations will focus on:

   (a) Determination of the mode of cellular entry (diffusion, facilitated diffusion, active transport);

   (b) efflux characteristics of the drug;

(c) determination of kinetic constants (Km and Vmax) for transport and their relationship to drug sensitivity; and,

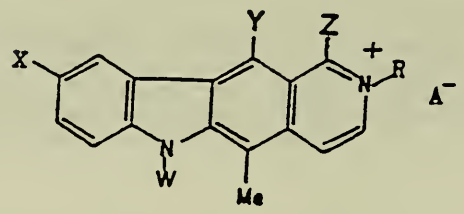
(d) identification of a possible physiological carrier responsible for selective uptake of the drug by CNS tumor cells.

2. An examination of the transport characteristics of the drug by normal human brain has recently been initiated and should provide information pertaining to the selectivity of the drug for normal vs. tumor tissue.

Publications

None

TABLE 1. SELECTIVE CYTOTOXICITY OF SEVERAL ELLIPTICINE ANALOGS TOWARD CENTRAL NERVOUS SYSTEM TUMOR CELLS IN VITRO



NSC	W	X	Y	Z	R	A	CNS Specificity
351710D	H	H	Me	H	Me	I	Yes
352299D	H	H	Me	H	Me	OMS	Yes
336003	H	H	H	Me	Me	OAc	+/-
353076D	H	H	Me	H	$CH_2CH_2NEt_2$-1/2HBr	Br	No
371106	H	H	Me	H	CH(Me)OCOOEt	Cl	No
371107	H	H	Et	H	CH(Me)OCOOEt	Cl	No
378695	H	H	CH_2O sugar	H	Me	Sugar RO^- (NH=NMe)	No
264137	H	HO	Me	H	Me	OAc	+/-
311152	H	HO	Me	H	$CH_2CH_2NEt_2$	OAc	No
371105	H	HO	Me	H	CH(Me)OCOOEt	Cl	No
155693	H	Meo	Me	H	Me	I	Yes
155694	Me	Meo	Me	H	Me	-	Yes
627505	H	Meo	Me	H	Me	OAc	Yes
632855	H	Cl	Me	H	Me	OAc	Yes

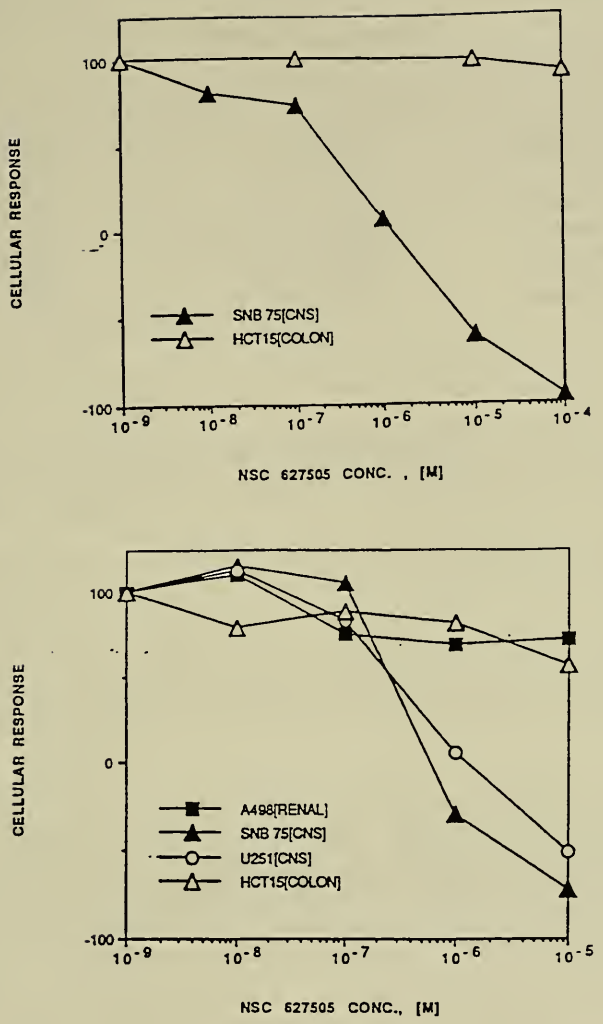


Figure 1. (TOP) Cytotoxicity of NSC 627505 Toward Central Nervous System Tumor Cell Lines (U-251 and SNB-75), the A498 Renal Cell Carcinoma and the HCT-15 Colon Adenocarcinoma Using the Sulforhodamine B Protein Assay. A value of 0 on the ordinate indicates cytostasis; a value of +100 indicates no drug effect while a value of -100 indicates that the drug produces nearly complete lethality.

(BOTTOM) Cytotoxicity of NSC 627505 Toward SNB-75 (CNS) and HCT-15 (Colon Adenocarcinoma)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07196-01 DDRD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Preclinical Pharmacological/Toxicological Evaluations of High Priority Compounds

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

PI:	Sherman Stinson	Pathologist	LDDRD NCI
Others:	Jean Keller	Bio Lab Tech	LDDRD NCI
	Angela Pittman	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI
	Louis Malspeis	Suprv. Research Chemist	LDDRD NCI
	David Vistica	Pharmacologist	LDDRD NCI
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COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION
Analytical Chemistry, Pharmacokinetics and Metabolism Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	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.)*

Appropriate animal models are being developed for initial in vivo pharmacological and toxicological evaluations of prototype compounds discovered in the primary in vitro anticancer and anti-AIDS drug screens. Development involves comparative studies of different animal species, vehicles and routes of administration to determine optimal model parameters for the specific drug. In addition, in collaboration with other LDDRD investigators, innovative in vivo techniques for the assessment of anticancer or anti-AIDS activity of compounds are being investigated.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—654-039

Objectives

The overall objective of this project is to facilitate the selection, prioritization and preclinical investigation of newly discovered prototype anti-HIV or antitumor compounds for accelerated development to rapid FDA-INDA approval and clinical trials. This is accomplished by developing and applying specific relevant animal model systems for exploratory preclinical pharmacology and toxicology studies. Specific aims include:

1. Determination of optimal animal species for exploratory pharmacological and toxicological experimentation.

2. Determination of feasible vehicles, routes, doses and/or schedule parameters for attaining relevant target-site drug concentrations.

3. Obtaining exploratory pharmacological and toxicological data to facilitate the optimal design of initial in vivo preclinical therapeutic evaluations and/or more comprehensive INDA-directed preclinical research and development.

4. Performance of detailed comprehensive or specialized toxicological evaluations if necessary to facilitate development of LDDRD priority compounds.

5. Collaboration with other LDDRD investigators in concerted pharmacokinetic, toxicological and experimental therapeutic studies to comprehensively support INDA preparation/submission and/or optimal design and execution of clinical phase I/II investigations for selected high-priority agents.

Major Findings

NSC 615985 (oxathiin carboxanilide) showed potent anti-HIV activity in the primary in vitro anti-HIV screen and the compound was recommended for high-priority preclinical development by the DCT Decision Network Committee. Comparative in vivo studies in hamsters and mice were initiated. Due to the poor solubility of NSC 615985 in vehicles suitable for iv use, and the rapid elimination of the compound when administered by this route, it was apparent that an alternative approach was necessary. Following solubility determinations in a wide variety of solvents, sesame oil was selected as the vehicle. In order to minimize potential metabolism by the liver and to achieve a prolonged systemic introduction of the compound, the sc route of administration was chosen for this initial study. Based on a theoretical one-compartment model, a dose necessary to achieve relevant drug concentrations was calculated. Single sc injections of 0.1 mmole/kg ^{14}C-NSC 615985 were given to mice and hamsters in 10 ml/kg sesame oil. Animals were bled at 1, 4 and 24 hours following injection, and plasma NSC 615985 concentrations were determined by tlc. Plasma concentrations of the drug reached 0.4-0.5 μM 4 hours following injection in both species. In hamsters, levels were approximately half these concentrations by 24 hours. In mice, however, the compound was barely detectable. As an estimate of relative

metabolism of the compound in these species, combined levels of metabolites was also determined. Plasma metabolites peaked after 4 hours in the mouse at 3 to 4 times the level found in hamsters. At 24 hours, levels in the mouse had dropped substantially, while in the hamster they had increased slightly. These studies confirmed preliminary plasma stability experiments suggesting that metabolism of NSC 615985 was more rapid in the mouse than in the hamster. They also indicated that prolonged plasma concentrations at least approaching the "target" concentration could be achieved in the hamster through use of the sc route of administration and an oil vehicle. However, since the concentrations achieved were only approximately half the target range, the drug concentration in the vehicle was at its maximum, and the injected vehicle volume was at reasonable limits, the prospects for administering a higher effective dose did not appear promising.

In an effort to overcome this problem, intragastric (ig) administration of the compound was attempted. Hamsters were intubated with the same dose-volume, and analyzed as described in the previous study. Significant levels of NSC 615985 were not detectable in the plasma through 24 hours. Levels of metabolites, however, were far in excess of those in hamsters or mice following sc administration. These results demonstrated that the ig route was not suitable for this drug, possibly due to rapid metabolism following absorption into the hepatic-portal circulation.

An alternative approach was to increase the solubility of the compound in the vehicle. To achieve this, NSC 615985 was first dissolved in DMSO at its maximum solubility, then 33 μl of this solution was added per ml of sesame oil to attain a concentration in the vehicle 3 times that of the original formulation (30 mmoles/l). The two formulations were then compared in an identical sc study to that reported above. The administered doses were 0.1 and 0.3 mmole/kg in 10 ml/kg vehicle. Using the new formulation, plasma concentrations exceeded the target concentration of 1 μM at 4 hours and were near 0.5 μM, still within the therapeutic range, after 24 hours. There were no overt signs of toxicity. As with the original formulation, levels of metabolites far exceeded those of the parent compound.

Having demonstrated that sustained therapeutic levels could be attained, a study was initiated to investigate if these levels could be maintained for a longer period without toxicity. Daily NSC 615985 doses of 0.3 mmole/kg in 10 ml/kg vehicle were given sc to hamsters for 8 days. Animals were bled 8 or 24 hours following selected injections and plasma samples were assayed by high pressure liquid chromatography (HPLC) for drug concentration. Comprehensive histopathology, blood chemistry and hematology were conducted at study termination. Minimum plasma concentrations (24 hour samples) of the drug exceeded the target levels of 1 μM after 2 days, and remained in excess for the duration of the study. Maximum (8 hour samples) and minimum levels plateaued between 3 and 4 days, indicating that an equilibrium state had been reached. No evidence of toxicity was observed, although significant pooling of oil in ventral sc areas was evident after 3 to 4 days.

Although sustained, "nontoxic" and "therapeutic" plasma concentrations of NSC 615985 could be achieved and maintained through use of a sesame oil formulation administered sc to hamsters at daily intervals, the high dose-volume required

for these effects was problematic. Converting the utilized dose (0.3 mmole/kg in 10 ml/kg vehicle) with relative surface area to body weight ratios for the hamster and humans indicated an equivalent human dose formulation would require 2.3 mmole in 78 ml vehicle for a 70 kg human, clearly an unfeasible situation. Therefore, an analog with better solubility and/or higher activity was sought.

A close structural analog of the compound, NSC 629243, showed a 10-fold higher activity (0.1 μM effective concentration) than NSC 615985. Formulation investigations conducted by extramural laboratories indicated that this analog also had better solubility characteristics in both aqueous and lipophilic solvents. Due to these properties, NSC 629243 was investigated further. Preliminary studies showed that the maximum solubility of NSC 629243 in sesame oil was 10-times higher (300 mmoles/l) than NSC 615985, and that decreasing the vehicle volume to 5 or 1 ml/kg body weight did not alter plasma concentrations appreciably when equivalent doses of the compound were administered to hamsters. Based on these findings, a study was initiated to investigate plasma concentrations following repeated dosing. Hamsters were given daily sc injections of 0.3 mmole/kg NSC 629243 in 1 ml/kg sesame oil for 8 days. Plasma samples were taken 8 and 24 hours following selected injections and analyzed by HPLC. Comprehensive histopathology, blood chemistry and hematology evaluations were performed at termination. Minimum plasma concentrations were approximately 1 μM when equilibrium was established. Maximum concentrations approached 2 μM. No evidence of toxicity or adverse side-effects was found.

This study demonstrated that plasma levels of NSC 629243 10- to 20-fold greater than the "target" concentration could be sustained for a prolonged period by sc injection of the drug in a sesame oil vehicle. The calculated equivalent human dose for this effect was 2.3 mmole in 7.8 ml sesame oil for a 70 kg human. Assuming that the *in vitro* anti-HIV activity is indicative of the concentration required for *in vivo* therapeutic efficacy, and that a linear relationship exists between administered dose and plasma concentration, then sustained therapeutic human levels (0.1 μM or above) would be achieved by daily sc administration of approximately 0.2 mmoles NSC 629243 in 0.7 ml sesame oil. This is a very reasonable scenario for clinical trials.

Methods

Drugs. The compounds NSC 615985 and 629243 were provided by the Uniroyal Chemical Company. The ^{14}C radiolabeled NSC 615985 used in these studies was obtained on contract and provided to us by the Drug Synthesis and Chemistry Branch, DTP, DCT, NCI.

Animals. The species used in these studies include CD2F1 mice (28 g), F344 rats (150g), and CR:RGH Syrian golden hamsters (120 g). All were males and obtained from the Animal Production Area NCI-FCRDC, Frederick MD.

Protocol: Comparative plasma levels in mice and hamsters. The dosing solution was prepared by dissolving ^{14}C-NSC 615985 in sesame oil at a concentration of 10 mmoles/l, with a specific activity of 9.4 mCi/mmole. A single sc injection consisting of 0.1 mmole/kg in 10 ml/kg sesame oil was given to 9 mice and 9 hamsters in the anterior dorsal midline. Nine additional hamsters were given the same dose and volume by ig intubation. Three animals from each treatment

group were exsanguinated via cardiac puncture 1, 4, and 24 hours following dosing, and heparinized plasma samples were collected. Plasma NSC 615985 concentrations were determined by tlc as described below.

Protocol: Comparative plasma levels with different drug concentrations. Hamsters were given a single sc injection of 0.1 or 0.3 mmole/kg in 10 ml/kg vehicle. Dosing, sampling, and analysis techniques were identical to the foregoing protocol. The solution for the lower dose group was prepared as above. The solution for the higher dose group was prepared by dissolving 300 mg ^{14}C-NSC 615985 in each ml DMSO, and adding 33 μl of this solution for each ml of sesame oil. The final concentration was 30 mmoles/l with a specific activity of 7.9 mCi/mmole.

Protocol: Repeated dose studies. Hamsters received daily sc injections of 0.3 mmoles/kg NSC 615985 or NSC 629243 at 24 hour intervals. The NSC 615985 dosing solution was prepared as above. The NSC 629243 concentration in its sesame oil vehicle was 316 mM, and the injected volume was 1 ml/kg. Dosing solutions were prepared fresh each day, immediately prior to injection. Groups of hamsters were anesthetized at selected intervals, and 0.5-1 ml of blood was collected from the suborbital sinus.

At study termination, treated hamsters, vehicle controls and 5 untreated hamsters maintained under the same conditions in the same facility were bled for chemistry and hematology studies as colony controls. Animals were sacrificed by anesthetization and exsanguination, and complete gross and microscopic necropsies were performed.

Thin-layer chromatography. This procedure was used for all NSC 615985 protocols involving radioactive material. NSC 615985 was added to heparinized plasma samples to a concentration of 50 μg/ml. Plasma (0.1 ml) was extracted three times with 0.3 ml of toluene. Samples were centrifuged following each extraction, the organic phases were combined and evaporated to dryness under a stream of N_2. The residue was dissolved in 20 μl of acetone and applied to silica gel plates. Plates were eluted for 10 cm with a solvent consisting of methylene chloride:ethyl acetate (9:1). The spots corresponding to NSC 615985 were recovered from the plates by scraping. Other eluted regions of the plates were recovered in 1-2 cm increments. Recovered fractions were suspended in scintillation fluid and the DPM quantitated. Values were calculated from the specific activity of the compound, or expressed as DPM. Aliquots of the extracted plasma samples were also counted and the total unextracted DPM were quantitated. Using this technique, recovery of compound from plasma was consistently 90-95 percent in the concentration range of 0.1-10 μM.

HPLC: NSC 615985. Samples were mixed vigorously for 1 minute with 1.2 ml toluene, centrifuged, and the toluene extract collected. This extraction procedure was repeated 2 additional times, and the extracts were combined. Extracts were evaporated to dryness in a Rotovapor RE 111 (Buci, Flaival, Switzerland). The evaporator flask was rinsed 3 times with 0.4 ml methanol, the rinses were combined and evaporated to dryness under nitrogen gas. The residue was dissolved in 100 μl methanol and 50 μl was injected on column. Analysis employed a modification of the HPLC method of Nomeir, et al. Chromatography was performed with a Waters model 600 E HPLC system and a Waters model 990

photodiode array detector. The system was equipped with a 4.6 mm x 250 mm stainless steel column packed with a 8 μm Dynamax-60A C_{18} stationary phase (Rainin Instrument Co., Woburn, MA). A guard column containing the same packing was directly coupled before the analytical column. Chromatography was effected with an isocratic eluent at 1.0 ml/min and concurrent UV detection of NSC 615985 at 259 nm and NSC 629243 at 283 nm. The mobile phase was acetonitrile:0.01 M, pH 4.5 potassium phosphate buffer (75:25, v/v).

HPLC: NSC 629243. Samples were mixed vigorously for 1 minute with 2.0 ml methanol, placed on ice for 10 minutes, centrifuged, and the liquid phase was collected and evaporated to dryness in a centrifugal vacuum concentrator (Jouan, Inc., Winchester, VA). The residue was dissolved in 100 μl methanol and 50 μl was applied on the column. Chromatography and analysis were identical to the methods described above.

Proposed Course

Studies are currently underway to find an analog of NSC 629243 which is not metabolized and has a longer half-life in vivo. Approximately 30 analogs are being evaluated in liver homogenates and in animal models to achieve this end.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07197-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Characterization of Human Tumor Cell Lines for Use in NCI Antitumor Drug Screen

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

PI:	Sherman Stinson	Pathologist	LDDRD NCI
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	Michael Boyd	Laboratory Chief	LDDRD NCI
	Michael Alley	Pharmacologist	LDDRD NCI
	Robert Shoemaker	Research Biologist	LDDRD NCI
	Philip Skehan	Cancer Expert	LDDRD NCI

COOPERATING UNITS *(if any)*

University of Freiburg, Department of Medical Oncology (Dr. H. Fiebig); Program Resources, Inc., Clinical Immunology Services, FCRDC (Dr. W. Kopp)

LAB/BRANCH

Laboratory of Drug Discovery Research & Development, DTP, DCT

SECTION

Analytical Chemistry, Pharmacokinetics and Metabolism Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	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.)*

Detailed characterizations of human tumor cell lines are being conducted to determine the suitability of individual cell lines for inclusion in the in vitro anticancer drug screen, as well as to aid in the evaluation of data from the screen. Characterizations include histological and ultrastructural observations and immunocytochemical testing with panels of antibodies directed against tissue and tumor specific antigens, and antigens associated with specific biological properties (multiple drug resistance, oncogene encoded products, etc.). The influences of the growth environment (in vitro monolayer or three dimensional, in vivo xenograft) and other cultivation parameters on expression of cell line characteristics are also being studied.

Objectives

The overall objective of this project is to utilize morphologic and immunocytochemical techniques to characterize human tumor cell lines derived from neoplasms representing major categories of human cancer. The purpose of these characterizations is to determine the suitability of individual cell lines for inclusion in specific disease-oriented panels of the NCI antitumor drug screen, and to define certain biological properties of these lines which will aid in evaluation of data from the screen. Specific aims include:

1. Confirmation of tissue of origin, tumor type and degree of differentiation of candidate human tumor cell lines.

2. Investigation of the expression by panel cell lines of features associated with drug resistance or sensitivity, and correlation of this with drug response data acquired from the anticancer drug screen.

3. Investigation of the expression by panel cell lines of oncogene-encoded proteins, and correlation of this with tumor type and characteristics, and with drug response data from the anticancer drug screen.

4. Investigation of the influence of various cultivation parameters on the expression of specific cell line characteristics.

Major Findings

Characterizations Related to Determining the Suitability of Individual Cell Lines for Inclusion in Specific Disease-Oriented Subpanels: Solid tumor cell lines which met initial quality assurance and assay suitability criteria were analyzed using histological, ultrastructural and immunocytochemical techniques to confirm their tissues of origin, and tumor types, and delineate various other features such as degree of differentiation and the expression of specific antigens associated with particular human tumor groupings. For immunocytochemical analyses, 50 different antibodies were used. These were grouped into panels recognizing antigens associated with different intermediate filament types and specific cytokeratin (CK) polypeptides (24 antibodies), and antigens associated with mucinous type adenocarcinomas (7 antibodies), melanomas (6 antibodies), urinary tract cancers (9 antibodies), and muscle tissue (4 antibodies). Leukemia and lymphoma cell lines were analyzed by flow cytometry for appropriate "cluster of differentiation" (CD) antigens as recognized by specific monoclonal antibodies. Information on the cell line origins and on other related studies are reported in detail in a manuscript (1). Recent results of immunocytochemical assays of specific CKs, which are not reported in the latter manuscript, are given in Table 1 of this section. A brief summary of all of these related studies for each subpanel follows. It should be emphasized that these results are illustrative for the current screening panel, and that similar studies are ongoing for many of the several hundred additional cell lines that the program has acquired for evaluation as potential candidates for future use in the screen.

While the initial effort to select the most representative lines for the various subpanels of those available to us at the time was predominantly empirical and based upon available "historical" documentation, it is now apparent that some of these are not particularly representative of their respective disease categories. In some cases this reflects the difficulty of deriving cell lines from particular types of human cancer. In other cases it may be due to cell line derivation from atypical tumors, or to selection by cell culture conditions of tumor cell subpopulations which do not express characteristic features of the tumor category. Whatever the cause of the problem, our characterizations have been invaluable in indicating the most appropriate selections, and in pinpointing panel areas which are in need of future development. They will continue to be a major selection factor when the screening panel is revised and/or expanded. In addition, subsets of tumor categories and groupings of cell lines from different tumor categories have been identified which should enhance our interpretations of drug screening data. Overall, these ongoing investigations have already clearly confirmed the essential value of comprehensive characterization for potential cell lines for the "disease-oriented" screening model.

Expression by Screening Panel Cell Lines of Features Associated With Drug Resistance or Sensitivity: P-glycoprotein is a high molecular weight membrane glycoprotein believed to act as a transmembrane pump. Its overexpression in tumors is associated with a form of multidrug resistance encompassing several diverse classes of commonly used anticancer agents. Its expression in panel cell lines was analyzed immunocytochemically using three antibodies recognizing different epitopes of p-glycoprotein, C219, JSB-1, and MRK-16. The techniques used and the antibody characteristics are described in the Experimental Design and Methods section. These studies are currently in progress, and the results reported here should be considered preliminary. Some cell lines showed a definitive reaction with all 3 antibodies including HCT-15, EKVX, CAKI-1, and the adriamycin-resistant sublines of MCF-7, K-562 and P-388 used as positive controls. DLD-1 and SKOV-3 showed a definitive reaction with one antibody and a weak reaction with another. UO-31, ACHN and SR showed a definitive reaction with only one antibody. COLO-205, KM-12 and OVCAR-4 each reacted weakly with a single antibody. Confirmatory immunocytochemical assays and final cell counts are in progress and data analysis is incomplete, but preliminary assessment indicates that a relationship between antibody reaction and resistance to adriamycin is present. There are, however, a number of lines exhibiting relative resistance to adriamycin which show no reaction with the antibodies. Correlation of antibody reaction data with cell line sensitivities to other drugs implicated in multidrug resistance is in progress.

Expression of Oncogene-Encoded Proteins by Panel Cell Lines: Epidermal growth factor receptor (EGFR). A domain of EGFR is encoded by the c-erbB oncogene. High expression is found in a variety of human cancers, and may be an important determinant of the transformed phenotype. EGFR expression was studied in panel cell lines immunocytochemically. High expression of EGFR was found in CNS line XF-498, colon lines DLD-1, HCC-2998, KM-20L2 and SW-620, lung adenocarcinoma lines EKVX, HOP-18, HOP-62 and NCI-H322, ovarian lines OVCAR-3 and OVCAR-5, and renal line CAKI-1. There was no apparent relationship between EGFR expression and degree of differentiation, growth rate or other characterized traits. These studies do, however, define a geneticallyrelated group of cell lines which may

also share common drug sensitivity patterns, and analysis of screening data for standard chemotherapeutic agents is underway to see if such patterns are present. If standard or new agents are discovered which target cell lines expressing high levels of EGFR, it would have important therapeutic implications, as the presence of this feature in human tumors can be easily tested by immunocytochemical and molecular biological techniques.

Methods

Cell lines and cell line cultivation. The 60 cell lines composing the current screening panel. Other cell lines analyzed, being analyzed or to be analyzed are too numerous to list here. Cell lines are grown in monolayer culture in RPMI 1640 medium supplemented with 5 or 10 percent fetal bovine serum and 2mM L-glutamine. Soft agar cultures are provided by Dr. Michael C. Alley.
When possible, cell lines are also grown by conventional techniques as xenografts in nude mice. Xenografts have all been grown subcutaneously with the exception of OVCAR-3 which was grown intraperitoneally.

For the study of extracellular matrix effects, cell lines are grown on a thin layer of Matrigel (Collaborative R, Inc. Bedford, MA), a solublized tissue basement membrane preparation containing laminin, collagen type IV, heparin sulfate, proteoglycan and enactin. Other culture conditions are as described above.

Immunocytochemical assays: The antibodies used in the initial characterizations, their sources and dilutions are reported in reference 1. Information on antibodies specific for individual CKs, p-glycoprotein, and various oncogene products are given in Table 2. Optimal dilutions of antibodies are determined by titration assay against known positive and negative control samples.

Cells are harvested for assays when 70-90 percent confluent by scraping, and were either centrifuged onto glass slides, or applied to teflon-coated slides with multiple uncoated assay wells. Alternatively, for desmin, myoglobin, myosin, actin and some GFAP (when cells are grown on extracellular matrix) assays, cells are grown directly on culture slides. Slides are air dried, and comparatively assayed unfixed or fixed in cold acetone. Assays are performed with commercially available kits using the peroxidase anti-peroxidase, avidin-biotin complex or alkaline phosphatase methods. All techniques show comparable sensitivity and results. Negative controls consisting of isotypic immunoglobulin are run for each cell line. Samples are prepared from a minimum of 2 separate cultures for each cell line for each assay. Solid tumor samples are snap frozen, and 5μ frozen sections are made.

Slides are evaluated microscopically at 400X. One hundred cells are counted from each of 3 separate fields. The percentage of positive cells is scored, and a relative staining intensity is qualitatively assessed on a scale of + to +++. Results from all fields and slides for a given cell line and assay are averaged and rounded to the nearest 5 percent. For some analytical purposes, results are expressed as a staining index, obtained as a product of the percentage of positive cells and the relative staining intensity.

Analysis of CD antigens by leukemia and lymphoma cell lines is performed by laser-based flow cytometry. These techniques are described in detail in publication 1.

Histology, histochemistry, and electron microscopy: Histological observations are made on cell line xeno/grafts prepared by standard techniques. Histochemical stains routinely done include alcian blue-PAS (for differentiation of mucopolysaccharides), Kreyberg stain (for keratin), Lilie stain (for melanin) and Bodian stain (for argyrophilic substances).

Electron microscopic examination is performed on cell line xenografts, soft agar colonies, and on cell pellets and embedded monolayers of cells grown in conventional culture. Sample preparation is done by standard techniques.

Proposed Course

This project is being terminated. Characterizations of newly acquired cell lines will be conducted under contract.

Publications

Stinson SF, Alley MC, Kopp WJ, Fiebig H-H, Mullendore LA, Kenney S, Keller J, Pittman A, Boyd MR. Morphological and immunocytochemical characterizatics of human tumor cell lines used in a disease-oriented anticancer drug screen. Cancer Res 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07198-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structure-Activity Optimization Strategies

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

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Others:	Michael R. Boyd	Laboratory Chief	LDDRD NCI
	Sherman Stinson	Pathologist	LDDRD NCI
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COOPERATING UNITS *(if any)*

Information Technology Branch, DTP, DCT (Dr. K. Paull); Laboratory of Molecular Pharmacology, DTP, DCT (Dr. E. Hamel); Drug Synthesis & Chemistry Branch, DTP, DCT (Dr. V. Narayanan); Pharmaceutical Resources Branch, DTP, DCT (Dr. R. Vishnuvajjala)

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Analytical Chemistry, Pharmacokinetics and Metabolism Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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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 in vivo efficacy of compounds identified to be active in the anti-AIDS and anticancer cell culture screens may be greatly influenced by their pharmacokinetics and metabolism. Chemical structural modification of a priority anti-HIV compound, NSC 629243, has been directed by pharmacokinetic and metabolic principles and studies in order to afford a structural analog or congener with greater in vivo efficacy than the original compound identified by the screen. Antitumor compounds selected on the basis of novel or otherwise interesting and strongly distinctive patterns of differential growth inhibition or cytotoxicity are referred by the DTP Screening Data Review Committee to DTP's Biological Evaluation Committee for Cancer for further investigation. Only a very small percentage of the compounds screened have been referred for further consideration. Among the large collection of other compounds, there are certain compounds which show more subtle or moderate activity profiles yet which potentially may have modes of action of interest for new drug discovery. Such structures could serve as the basis for a lead-directed synthetic program which seeks to prepare structural analogues with enhanced activity. Using the Compare pattern-recognition algorithm, new lead synthetic compounds with chemical structures which are not known to be tubulin active have been identified. Molecular modeling has been employed to design potential new tubulin active compounds.

Objectives

The in vivo disposition characteristics of drugs active in the cell culture screens to the drug discovery programs are used to guide the synthetic efforts to the development of compounds with optimal in vivo efficacy. Active compounds which are identified in screens may be so rapidly and extensively metabolized and excreted in vivo that adequate or sustained levels cannot be achieved in the body. The in vivo activity of such compounds may not exhibit activity considered adequate for further development consideration. In this project, structural modifications are undertaken with the potential of obtaining a compound which is also significantly active in vivo guided not only by the in vitro biological activity but also by in vivo pharmacokinetic and metabolism characteristics.

1. Employment of pharmacokinetic and metabolism considerations to design for subsequent synthesis of potentially improved analogues and congeners of selected new lead compounds, both synthetic and natural products, identified by the NCI/DTP in vitro anti-HIV or antitumor primary screens.

2. Investigation (e.g., by use of computerized pattern-recognition algorithms) of the new DTP/NCI in vitro primary antitumor screen as a novel and powerful new qualitative and quantitative bioanalytical tool to support: a) chemical analog development (the identification of "improved", but chemically related derivatives sharing the desired common mechanism of action) and, b) biological analog development (the identification of new structural classes with a desired common mode of action).

Methods/Major Findings

Analogue Development Based Upon Pharmacokinetic and Metabolic Considerations: Identification of the anti-HIV activity of the Uniroyal compounds NSC 615985 and NSC 629243 led to research conducted in LDDRD which is expected to move NSC 629243 to a clinical trial. The selection of NSC 629243 for further development was based upon both antiviral and solubility considerations. Both compounds were hydrolytically cleaved to inactive product(s) in plasma, and both were shown to be more stable in hamster plasma than in mouse plasma. In vivo studies in the hamster demonstrated that both compounds were extensively metabolized and that plasma levels of metabolites greatly exceeded that of the parent drugs. The structures of the metabolites have not been identified. Despite the extensive metabolism, the subcutaneous injection of the compounds in sesame oil to hamsters afforded plasma levels of the compounds which were equal to or greater than the concentration required for antiviral activity in vitro.

The in vivo data regarding the metabolism of the compounds directed attention to the need to evaluate structural modification of the molecules which would result in compounds which were more resistant to in vivo metabolism and which would substantially retain the antiviral activity. A program of structural modification was initiated which focused upon achieving this objective. A priori consideration of the structure of NSC 629243 suggested that metabolic inactivation is primarily the result of cleavage of the isopropyl ester and to

a lesser extent, the thiocarbamate moieties. Other LDDRD studies showed that the carboxylic acid corresponding to NSC 629243 was devoid of anti-HIV activity.

Initially, compounds were prepared (by Uniroyal chemists) in which the ester moiety was retained and the thiocarbamate region of the molecule was altered. The anti-HIV activity of the compounds synthesized demonstrated that with suitable modification there was good retention of activity. The synthetic efforts were then centered upon compounds in which the thiocarbamate moiety was retained and structural alterations were in the ester moiety. The antiviral activity of the analogues derived from these studies permitted the design of structures which were expected to be active and to be cleared more slowly from the body than NSC 629243. Examination of the in vitro metabolism of these compounds employing hamster liver homogenate demonstrated that selected compounds in which anti-HIV activity was retained, were more stable to metabolism than NSC 629243. The synthetic effort was guided by both the in vitro anti-HIV activity and in vitro metabolic studies. Studies concerned with characterizing the murine i.v and p.o. pharmacokinetics of the lead compounds in comparison with that of NSC 629243 are in progress.

Identification of New Synthetic Compounds Targeted to the Cellular Microtubule System: A goal of this project was to find new lead synthetic compounds with chemical structures which were not known to be tubulin active and to utilize these structures for the development of a drug synthesis effort. There has been recent renewed interest in cytotoxic agents which are targeted to the microtubule system forming the structure of the mitotic spindle. Many antimitotic compounds have been evaluated clinically, e.g. colchicine, the podophyllotoxins, vincristine, vinblastine, maytansine and taxol. At present, the tubulin active compounds which have been brought to clinical trial are natural products. Taxol, NSC 125973, is a mitotic spindle poison which inhibits mitosis, however, taxol differs from other spindle poisons by enhancing polymerization of the tubulin units and forming unusually stable microtubules. Responses in patients recently observed in phase II trials has prompted interest in significantly expanded trials. The need for an adequate supply of material for these trials has been a matter of considerable concern to DTP.

DTP/NCI currently has interest in the clinical development of one of the antimitotic dolastatins. The dolastatins are a series of peptides isolated by Pettit et al. from the sea hare, Dolabella auricularia, a marine animal found in the Indian Ocean. At present, dolastatin 10, an unique linear pentapeptide, is the most potent member of the series. The compound inhibits mitosis in cells in culture and the microtubule assembly in vitro and was shown to be one of the most tubulin-active compounds known. The 50% growth inhibition response of the panel of human tumor cell lines of the anticancer screen to dolastatin 10, was employed as a seed using Compare against the entire database of synthetic and natural product compounds tested to date. For each compound in the reference database, the difference between the mean response of all the cell lines and that of a specific cell line was calculated and the difference for each cell line was compared with that of the compound used as the seed. The statistics compared the average of the differences for each compound to that of the seed and the tabulated correlation coefficients were computed from these differences.

From the database of screened synthetic compounds, the Compare programs afforded those 100 compounds giving the highest correlation coefficients. Among these, a significant fraction were dolastatins or compounds derived from extracts of marine animals and approximately one-third were synthetic compounds unrelated to established tubulin binders. The latter group included a set of compounds which were analogues and represented a class of compounds which were not previously known to have tubulin activity. When these compounds were employed as seeds in the Compare analysis of the Standard Agent database, at the growth inhibitory levels, GI50, and at the cytostatic levels, TGI, the highest correlations were with compounds known to be tubulin active. Experimental evaluation, by Dr. Ernest Hamel, LMP, DTP, DCT, NCI, established that these compounds are tubulin active. These studies support the concept that the identified synthetic agents may serve as basic structures for a new lead-directed drug synthesis effort.

Proposed Course

To help achieve the objective of this project we will regularly review the in vitro activity of compounds structurally related to those identified as active in the anti-AIDS and anticancer screens to attempt to optimize in vivo efficacy by initiating the preparation of compounds with potentially improved disposition or metabolic attributes. Comparative animal pharmacokinetic studies will be undertaken in order to monitor achievement of the objective. Computerized pattern-recognition algorithms, such as exemplified by the Compare programs developed by Dr. Kenneth Paull in collaboration with LDDRD investigators and other DTP and DCT staff, facilitate comparisons of the spectrum of in vitro antitumor activity of a given seed compound relative to the database of all compounds screened, or any desired subset thereof. Such algorithms can help identify (a) compounds which exhibit a spectrum of antitumor activity against the panel of cell lines which is similar to other compounds which may share common biological properties and (b) compounds whose spectrum of activity against the panel of cell lines is dissimilar from those which are known. We will attempt to use pattern-recognition algorithms to help exploit the new in vitro screen as a tool to guide molecules with desired modes of action, or to identify new compounds, or previously known compounds having novel biological mechanisms relative to current "standards". An initial step in these studies is to use the Compare programs to search the screening databases for synthetic compounds whose spectrum of in vitro activity (and, by implication, potential mode of action) is similar to that of complex natural products which are difficult to obtain or are in short supply; another step is to search the databases for compounds having unique modes of action. The structural identity of the compounds will serve as the basis for synthetic efforts which will be proposed.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07199-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Approaches to Assessment of Experimental Metastatic Tumor Burden

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

PI:	Robert Shoemaker	Biologist	LDDRD NCI
Others:	Wu Lin	Visiting Fellow	LDDRD NCI
	Anne Smythe	General Fellow	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Cell Biology, Biochemistry and Experimental Therapeutics Section

INSTITUTE AND LOCATION

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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.)*

Experimental therapeutic models require the availability of sensitive and quantitative methods for assessing tumor burden and response to therapy; conventional approaches typically involve direct macroscopic or microscopic measurement of tumor mass. We are exploring the application of molecular biologic approaches to development of new in vivo metastatic, orthotopic and other xenograft models where more precise quantitative determination of tumor burden is critical. Utilizing probes for repetitive human DNA sequences, we have demonstrated that human tumor DNA can be detected in DNA extracted from whole mouse organs and that this signal can be utilized to characterize the time-course and tissue distribution of metastatic cells. Future work will be directed towards improving methods of dot-blot quantitation, application of the polymerase chain reaction to enhancement of sensitivity for detection of human DNA sequences and application of DNA probe technology to evaluation of therapeutic effects.

Objectives

This project is directed towards development of molecular approaches to detection and quantitation of human tumor DNA in experimental animals. There are three specific aims of this work:

1. Development of molecular methods for detection/quantitation of human tumor DNA admixed with mouse DNA.

2. Application of methods to characterization of tumor behavior in athymic mice.

3. Application of methods to measurement of therapeutic effects.

Methods/Major Findings

Identification of a Suitable Probe for Detection of Human DNA: To maximize sensitivity, primary consideration in selecting a DNA probe was given to DNA sequences present in very high copy number in the human genome. The Alu family of repetitive DNA sequences is found scattered in introns throughout the human genome and represents approximately 5% of the total DNA. A commercially available Alu probe (Oncor, Gaithersburg, MD) was selected for use in the preliminary studies described here.

Detection/Quantitation of Human DNA Admixed with Mouse DNA: A dot-blot hybridization assay was developed using the Alu probe and calibrated using known amounts of human DNA. Scanning densitometry of dot-blots indicated a linear signal for human DNA (either LOX or K562 DNA) up to approximately 10 ng of DNA. In order to assess the sensitivity of this assay to interference by mouse sequences, in vitro mixing experiments were performed employing mouse lung DNA admixed with human tumor cell DNA. Under the high stringency conditions used for hybridization (42°C, high salt, and 45% formamide), amounts of mouse DNA up to 1000 ng did not interfere with detection of human DNA in the 1-10 ng range.

Application of DNA Probe Technology to Characterization of the Tissue Distribution of Metastatic Human Tumor Cells in Athymic Mice: To evaluate the potential for detection of human tumor DNA in mouse host tissue in vivo, an experiment was set up in which a group of young female athymic mice was inoculated subcutaneously with 1×10^7 LOX melanoma cells, a line which we have shown to spontaneously metastasize to lungs with high efficiency (Shoemaker RH, Dykes D, Plowman J, Abbott BJ, Mayo JG, Fodstad O, Boyd MR: Cancer Research 51: 2837-2841, 1991). Animals were killed at intervals over a four week period and peripheral blood, lung, axillary lymph node, liver, spleen, brain, and tumor tissue were obtained for histological analysis and DNA extraction. Preliminary dot-blot analysis of the terminal samples indicated the presence of metastatic tumor cells in peripheral blood, spleen, lymph node, and in the lungs. Preliminary review of the corresponding histological samples indicated large numbers of metastatic cells in the lungs and isolated clusters of tumor cells in the spleen. Further analyses of samples from this experiment are currently in progress.

Application of DNA Probe Technology to Evaluation of Therapeutic Effects in Treatment of Established Pulmonary Metastases: Using the LOX melanoma model, an experiment was performed to evaluate the potential of the human DNA assay for assessment of therapeutic effects in treatment of established pulmonary metastases. Animals were inoculated subcutaneously with 1 x 10^7 tumor cells, staged for a week to allow formation of pulmonary metastases, and treated by intraperitoneal injection with cyclophosphamide (100, 50, 25, or 0 mg/kg) on days 8, 12, and 16 after tumor cell inoculation.

Measurements of subcutaneous tumors were taken throughout the experiment. Half of the animals were killed on day 21 and the remainder on day 28. Animals were necropsied, and lung tissue was removed for histological evaluation and human DNA assay. At the 100 mg/kg dose, complete regressions were obtained in all animals. This strong therapeutic effect was expected on the basis of the high degree of *in vitro* sensitivity of LOX to alkylating agents and on the results of *in vivo* studies using a bioassay endpoint. Preliminary studies have indicated that the underlying basis for this sensitivity is a defect in DNA repair capability (Mer^- DNA repair phenotype). Gross observations of lungs removed from animals treated at the 100 mg/kg dose suggested that pulmonary metastases had been eradicated as well. Metastatic lesions were grossly visible in animals treated at lower doses and in the controls. Histological and DNA analyses of samples from this experiment are currently in progress.

Proposed Course

Results from preliminary studies suggest that molecular methods directed towards detection of repetitive human DNA sequences in tissue samples from athymic mice will be useful for studies of tumor xenograft biology as well as measurement of therapeutic effects. The dot-blot hybridization assay employing the Alu probe provides a simple and rapid method for detection and measurement of human DNA in mouse tissues. The following section outlines studies designed to further develop this technology and apply it in key areas of *in vivo* model development.

Detailed Studies of the Time-Course and Tissue Distribution of Metastatic Human Tumor Cells in Athymic Mice: The LOX melanoma provides a useful model for the detailed examination of the distribution of metastatic cells over time. Cells of this tumor line metastasize reproducibly to the lung following subcutaneous inoculation into athymic mice and produce lethal disease. As indicated above, preliminary studies utilizing DNA probe technology have indicated the presence of tumor cells in tissues where even micrometastases have not been detected using conventional technique. We plan to pursue this line of investigation to define the pattern of distribution of metastatic LOX cells in detail. As other tumor cell lines with the potential for spontaneous metastasis are identified, these will be investigated as well.

Comparison of Relative Sensitivity of Bioassay, Human DNA Assay, and Histological Analysis in Detection of Pulmonary Metastases: The DNA dot-blot assay appears to have considerably more sensitivity than histological methods for detection of human tumor metastatic lesions in athymic mice and has obvious advantages for quantitation. We would expect that the bioassay approach may have comparable sensitivity to the DNA assay. To formally define the relative sensitivity of

these methods we will inoculate a group of mice with the LOX melanoma and then sample lung tissue at intervals for the various analyses.

Survey of Metastatic Potential of Human Tumor Cell Lines Used for In Vitro Screening: In order to evaluate the metastatic potential of cell lines in the current in vitro drug screening panel, animals will be inoculated subcutaneously with cells, staged for various intervals then sacrificed in groups over time for evaluation of potential metastatic target tissues (lung, liver, brain, lymph nodes) for human tumor DNA. As mentioned above, there seems to be an excess representation of melanomas among published accounts of spontaneous metastasis of human tumors in athymic mice. For this reason, and in the interest of building upon the only currently available practical metastatic model (LOX melanoma), we will initially focus on characterization of metastatic potential of the melanoma cell line panel. One of the lines in this panel, M14, has previously been reported to give rise to sublines which can form spontaneous metastases in young nude mice and will be the focus for initial work. Next in priority will be tumor lines where micrometastases have been observed histologically in the course of routine tumorigenicity studies and those with an invasive pattern of subcutaneous growth, a factor which we have previously found to correlate with metastatic potential (Gazdar AF, Shoemaker R, Mayo J, Oie HK, Donovan P, Fine D. In: Rygaard J, Brunner N, Graem N, Spang-Thomsen M (eds.): Immune-Deficient Animals in Biomedical Research, Basel, Karger, 1987, pp. 277-280.). Several of the carcinoma lines fall into this category. This work will be integrated with efforts to select cell lines with increased metastatic potential.

Refinement of Methods for Quantitation of Therapeutic Effects in Orthotopic and Metastatic Models: Preliminary results suggest that extraction of whole-organ DNA from sites of metastasis and quantitation of human DNA using the dot-blot hybridization assay may provide an acceptable method for evaluating tumor burden and response to treatment in experimental therapeutic studies. Detailed studies will be pursued to firmly establish this and to define the relative utility of this approach compared to the conventional endpoints (survival or bioassay). In relation to orthotopic transplantation models of lung and brain cancer, we will evaluate the DNA probe technology for measuring lung or brain tumor burden in whole-organ extracts. As in the case of metastatic models, appropriate comparisons will be made with conventional approaches to measurement of therapeutic effects.

Development of Methods to Enhance Sensitivity for Detection of Human DNA: A variety of approaches are available to increase the sensitivity of the dot-blot assay. The most powerful of these is use of the polymerase chain reaction (Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Science 239: 487-491, 1988). Using the polymerase chain reaction (PCR), amplification of DNA on the order of 10^5-fold can be achieved routinely. We will evaluate PCR-amplification of Alu sequences as a means of detecting very small numbers of human tumor cells in athymic mouse peripheral blood and potential metastatic target organs.

One of the most critical areas in this project will be design of primers which will not hybridize to the murine B1 family of repetitive DNA sequences. This family shows a high degree of sequence homology with the human Alu family and

makes design of specific primers somewhat challenging. However, the sequences of both families are known and available in the Genbank database. Furthermore, Alu-specific primers have been reported recently for studies of genetic polymorphism (Sinnett D, Deragon JM, Simard LR, Labuda D., Genomics 7: 331-334, 1990). We will utilize this information to design and optimize primers for the present application. Yields of human DNA in the PCR product will be measured using the dot-blot assay.

Publications

Shoemaker RH, Dykes D, Plowman J, Abbott BJ, Mayo JG, Fodstad O, Boyd, MR. Practical spontaneous metastasis model for in vivo therapeutic studies using a human melanoma. Cancer Research 1991;51:2837-41.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07300-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Investigation of a Link Between Anti-HIV and Phorbol Receptor Binding Activities

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

PI:	John Cardellina II	Supvr. Research Chemist	LDDRD NCI
Others:	Richard Fuller	Chemist	LDDRD NCI
	John Beutler	Cancer Expert	LDDRD NCI
	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI
	Mark Tischler	Visiting Fellow	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI (Dr. P. Blumberg); Institut fur Biochemie der Universitat zu Koln, Germany (Dr. F.-J. Marner); Research Triangle Institute, Research Triangle Park, NC (Dr. M. Wall); Brigham Young University, Provo, Utah (Dr. P. Cox)

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Natural Products Chemistry Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	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.)*

The isolation and identification of prostratin as an anti-HIV compound and the observation of a significant number of anti-HIV active extracts from the Euphorbiaceae led us to consider a linkage of phorbol receptor binding activity with AIDS antiviral activity. This concept of linkage has been supported by observation of anti-HIV activity in the aplysiatoxins and indolactams. A very small number of plant extracts outside the Euphorbiaceae are active in the phorbol receptor binding assay and the anti-HIV screen; from these leads we have discovered anti-HIV active triterpenes and xanthophylls.

Personnel (cont'd)

James McMahon	Biologist	LDDRD NCI
Tawnya McKee	IRTA Fellow	LDDRD NCI
Laurent Decosterd	Visiting Fellow	LDDRD NCI

Objectives

The emergence of a significant number of anti-HIV leads in extracts of plants of the Euphorbiaceae and Thymelaceae from the DTP's AIDS antiviral screening program and the subsequent isolation of prostratin as the primary anti-HIV constituent from Homalanthus acuminatus led us to consider the possibility of a link between HIV inhibitory and phorbol receptor binding activities. This concept of linkage was bolstered when anti-HIV activity in a blue-green algal extract was traced to aplysiatoxin, a known protein kinase C agonist. As a consequence of these observations, we embarked on a project with the following objectives:

1. Examination of the relationship between phorbol receptor binding and AIDS-antiviral activities in various chemotypes known to be active at the phorbol receptor.

2. Evaluation of the potential of this phorbol dibutyrate (PDBu) binding assay to serve as a rapid screen for bioactivity guided fractionation.

3. Search for novel anti-HIV/phorbol receptor binding agents by screening extracts of microbes, marine organisms and plants outside the Euphorbiaceae/Thymelaceae in both assays.

4. Utilization of the PDBu assay in dereplication studies of extracts active in both assays.

Methods

1. PDBu binding assay screening of plant extracts outside the Euphorbiaceae.

2. Dereplication of HIV-inhibitory extracts of Euphorbiaceae.

3. Investigation of members of the Euphorbiaceae.

Major Findings

Work on this project has advanced on several fronts.

PDBu Binding Assay Screening of Plant Extracts Outside the Euphorbiaceae

Approximately 60 plant extracts from families other than the Euphorbiaceae and Thymelaceae have been evaluated in the phorbol receptor assay. Six genera

exhibited some level of activity at the crude extract stage; of these, two failed to reproduce activity in one or both assays after an initial fractionation step. The remaining four leads have been investigated in some detail:

1) Combretum bracteatum - Activity in both assays was tracked conclusively to the xanthophyll lutein. No reference to lutein or any other carotenoid as active at the phorbol receptor could be gleaned from a search of the literature.

2) Anthocercis littorea - Fractionation of the limited amount of extract available resulted in concentration of both anti-HIV and PDBu activity in very minor, still impure fractions. Recollection efforts are underway.

3) Pothomorphe peltata - Fractionation has led to an inactive prenylated catechol and an HIV-inhibitory dimer of that catechol. Noteworthy here is the curious divergence of the phorbol binding activity -- the monomeric catechol is weakly active in the PDBu assay (IC_{50} ~ 20μg/mL), while the dimer is inactive. The weak activity of the monomer was detected in the crude extract because the monomer comprises a significant portion (>3%) of the extract mass.

4) Iris sp. Two species of Iris, I. pseudacorus and I prismatica, were active in both assays. Initial efforts to fractionate I. pseudacorus resulted in a dramatic loss of biological activity in both screens. At this juncture, our attention focused on multiple reports in the recent literature on very unusual, oxidized and highly unstable triterpenes from Iris. We entered into a collaboration with Dr. Franz-Josef Marner and proceeded to test some of his extracts and pure compounds. Our supposition that the triterpenes isolated in the Marner laboratory were responsible for the activity in both assays was confirmed.

Dereplication of HIV-Inhibitory Extracts of Euphorbiaceae

Several extracts of representatives of the Euphorbiaceae were selected for chemical analysis and dereplication studies. Examples are given below.

1) Jatropha curcas. This is Samoan ethnobotanical material, provided by our collaborator, Dr. Paul Cox. Since only a minimal amount of extract was available and there is a plethora of literature reports on the chemistry of this genus, the logical course of action was to pursue the lead from a dereplication standpoint. Application of batch elution chromatography to concentrate the activity and subsequent microscale HPLC analysis, using a tandem of diode array and PDBu assay detection pinpointed the active material. Hydrolysis, acetylation and GC-MS analysis will be employed to characterize the active components by comparison to known phorbol diterpene standards.

2) Maprounea africana. The extract of the roots of this tree was highly cytotoxic, but HIV protective, and a strong phorbol receptor binder (IC_{50} 6 μg/ml). The activity was tracked through several chromatographic steps to a multiply functionalized triterpene (7B-hydroxy-3B-p-hydroxybenzoyl maprounic acid) reported by Walls's group from this tree. A more rigorous analysis of this material, however, revealed trace contamination by a mixture of exceedingly potent (PDBu assay) compounds. While the complexity of the mixture and the small sample size precluded purification and characterization of the individual

components, alkaline hydrolysis and acetylation of the mixture gave a product mixture dominated by a compound giving a GC-MS profile virtually identical to that obtained by identical treatment of mezerein, a daphnane diterpene. The pure triterpene, while inactive against HIV, is active in the 9PS assay (IC_{50} 0.056 μg/ml) and will be evaluated in the NCI human tumor cell line panel.

Investigation of Members of the Euphorbiaceae

The intent here is to investigate HIV-inhibitory extracts in an effort to uncover either anti-HIV phorbol receptor binding agents which are not potent tumor promoters or irritants, or anti-HIV agents which are not active at the phorbol receptor.

1) Excoecaria guineensis. Detailed analysis of these extracts has traced HIV-inhibitory activity to the xanthophyll lutein, a series of conjugated alkenamides and fractions containing mixtures of lipophilic diterpene esters. The phorbol receptor binding activity was associated with lutein and the diterpenes, but not the alkenamides.

2) Cyrtogonone argentea. An investigation of this previously unstudied genus has proceeded through only a few early chromatography steps; terpenoids appear to be abundant in the active fractions, but no individual constituents have yet been purified.

Proposed Course

Future work in this area encompasses both chemical and biological directions. Regarding the Euphorbiaceae, the natural products studies with Excoecaria, Jatropha and Cyrtogonone will be completed and other genera, previously unstudied chemically but active in the AIDS antiviral screen, will be investigated. Included in this group are Trigonostemnon, Fahrenheitia, Crotonogyne, Eremocarpus and Duvigneaudia. Extracts of these genera are potent binders to the PDBu receptor and exhibit some level of cytotoxicity and/or antiviral activity.

Among the leads outside the Euphorbiaceae, the HIV-inhibitory components of Anthocercis will be isolated and identified following recollection of the plant. We will continue to study the HIV-inhibitory and PDBu activities of Iris in collaboration with Dr. Marner. He will continue to provide extracts from numerous species of Iris for screening; in addition, he will provide samples of all of his isolates for bioassay. He will also provide expert guidance on the proper procedures for handling the extracts and conducting the isolation work, so that additional quantities of these interesting compounds can be produced as needed at NCI for secondary biological evaluation.

We will continue to screen extracts outside the Euphorbiaceae and Thymelaceae for activity in the phorbol receptor binding assay. In particular, we will test those extracts active in the human tumor cell line panel, since activity in the PDBu assay not only may give clues to the mode of action to be encountered, but it might also permit us to follow the activity more expeditiously to purified active metabolites.

In collaboration with Dr. Peter Blumberg, we will evaluate the behavior of lutein, the Iris triterpenoids and the prenylated catechol from Pothomorphe peltata on protein kinase C and in a number of other protocols in the Blumberg laboratory (binding of epidermal growth factor, release of arachidonic acid metabolites, induction of ornithine decarboxylase, edema formation, induction of hyperplasia and tumor promotion).

We will also provide quantities of these compounds to Dr. McMahon for a full in vitro evaluation of their HIV-inhibitory activity. He will further explore the mechanism of action of compounds active in both the anti-HIV screen and the phorbol receptor assay. A common thread appears to be impact on the calcium pathways.

Dr. McMahon has observed a consistent spiking, or enhancement, of viral p24 and reverse transcriptase production at the lowest protective doses in a number of phorbol esters. We plan to examine extracts active in both the anti-HIV and PDBu assays for production of p24 and reverse transcriptase. The goal is to identify extracts which do not exhibit this enhancement phenomenon, since they would be more likely to yield novel anti-HIV or PDBu receptor active compounds.

Publications

Gustafson KR, Cardellina JH II, McMahon JB, Gulakowski RJ, Cragg GM, Boyd MR, Ishitoya J, Szallasi Z, Lewin NE, Blumberg PM, Weislow OS, Beutler JA, Cox PA. A non-promoting phorobl from the Samoan medicinal plant, Homalanthus acuminatus, inhibits cell killing by HIV-1. J Med Chem 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07301-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Preparative Separation of Complex Mixtures of Priority Natural Products

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

PI:	John H. Cardellina II	Suprv. Research Chemist	LDDRD NCI
Other:	Richard Fuller	Chemist	LDDRD NCI
	Mark Tischler	Visiting Fellow	LDDRD NCI
	Tawnya McKee	IRTA Fellow	LDDRD NCI
	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI
	John Beutler	Cancer Expert	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Natural Products Chemistry Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	1.3	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.)*

Development of bulk isolation and purification methods for natural products from crude extracts and fractions is a crucial element for drug development. A variety of unique approaches have been adapted to the problems encountered in current work. Examples include normal phase HPLC on cyano-(taxol) and amino-(alkaloids) bonded phase columns and countercurrent chromatography (alkaloids).

Objective

Within the LDDRD, the Natural Products Chemistry Section is charged with isolating and identifying bioactive (antitumor and antiviral) compounds from extracts of microbes, plants and invertebrates. Since pharmacologically active natural products are all too frequently minor metabolites, efficient separation chemistry is crucial to achieving this program's goals. An important and continuing objective of our research is, therefore:

To develop, adapt and apply novel strategies for the isolation and purification of bioactive natural products from complex mixtures.

Methods

Chemical research in natural products chemistry is comprised of two major endeavors - isolation and identification. The identification of organic compounds is generally the principal area of focus, because it comprises the means of reaching the final goal. The last two decades have witnessed an incredible development of spectroanalytical techniques for structure elucidation; structures are now determined almost exclusively by spectroscopic means, with little or no reliance on chemical degradation or modification. Separation science has enjoyed a parallel phase of rapid evolution during the same period, but the emphasis has been on qualitative and quantitative analysis, rather than preparative or large scale separations. Natural products chemists have not been as quick to adapt new separation technology as they have new spectroscopic approaches. Consequently, natural products chemistry has reached a point where the tools exist to solve virtually any structure, as long as the compound can be purified from the original extract.

Adsorption chromatography has long occupied a preeminent role in the isolation of natural products. Certain well known characteristics of silica gel, such as relatively low costs and relatively high resolving capacity (selectivity), have allowed it to endure, while newer technology, such as pressurized (high, medium or low pressure) and vacuum chromatography have maintained its popularity. However, silica is not without drawbacks; irreversible adsorption and silica catalyzed hydrolytic and oxidative degradations have been frequent problems. As natural products chemists have begun to focus more intently on trace, potently bioactive but chemically labile molecules and on more polar metabolites, the unacceptable aspects of adsorption chromatography have become more evident. A number of alternatives to adsorption chromatography exist, but most have been developed as analytical or biochemical tools. Examples include size exclusion, or gel permeation, chromatography, bonded phase chromatography (HPLC or MPLC) and countercurrent chromatography (droplet or centrifugal).

Gel permeation, in theory, offers the advantage of minimal interaction between solute and solid support, as well as a short residence time. Its appeal is broadened by the availability of partition and, to some extent, hydrogen bonding or other surface interactions with solute molecules; these effects can be

enhanced or mitigated by judicious (or serendipitous) selection of solvent combinations.

Bonded phase chromatography also offers the advantage of reduced to non-existent adsorption, since the silylhydroxy groups of silica are blocked by a chemically bonded phase. Various bonded phases are available, ranging from the very non-polar, lipophilic C_{18} to very polar phases like the diol and aminopropyl types. These phases operate principally in a partition mode, and the more polar phases also retain solutes by hydrogen bonding mechanisms.

Droplet (DCC) and centrifugal (CCC or CPC) countercurrent chromatography are recent technological innovations that have revived liquid-liquid partitioning as a separation method and elevated it to a high resolution technique. This rapidly evolving technology is quickly gaining acceptance, but has thus far been utilized primarily for very polar molecules, since nearly all the two phase solvent systems contain water. There are now indications that more nonpolar compounds are amenable to separation by CCC with biphasic, all organic solvent systems.

The need for separation methodology with high selectivity and resolving capacity under chemically mild conditions is amply illustrated by the challenges inherent in purifying individual bryostatins from the complex mixtures of these minor metabolites which occur in the crude extracts of the producing organism. This is not an isolated example, or one restricted to marine natural products. There are numerous such examples from microbial, plant and marine natural products; in almost every case, the isolation and purification effort is the rate limiting step in a project. The requirement to develop efficient, large scale isolation strategies for such compounds as they advance forward to clinical development adds a new dimension to the problem.

Major Findings

We have approached some of our recent separation problems with a view toward exploring the application of unusual or novel chromatographic techniques. We have also sought to apply specific new techniques to existing problems.

An example of an existing problem is taxol, a highly functionalized antimitotic diterpene from the bark of trees of the genus Taxus. Taxol, which occurs as a minor component in an exceedingly complex matrix of related diterpenes, is required in increasingly larger quantities for clinical trials. Current protocols for isolation of taxol include partitioning and precipitation steps combined with a series of silica gel chromatographies. The crux of the problem has been the difficulty in separating taxol from its close structural analog, cephalomannine. Silica gel gives a rather poor yield and requires very polar solvent combinations, while reversed phase (C_{18}) approaches work well on an analytical level, but are difficult to scale up to preparative levels. The somewhat unusual approach of using a cyano bonded phase column in a normal phase elution mode has proven quite effective in separating a series of closely related, highly functionalized briaran diterpenes. This approach seemed, therefore, to offer considerable potential to solve the taxol/cephalomannine problem. Excellent resolution of the two compounds was achieved on a preparative

column operating under isocratic conditions; the solvents used are non-halogenated, relatively inexpensive and easy to evaporate.

We have recently encountered a number of different classes of alkaloids in our efforts to discover new anti-HIV agents. While each compound type is beset by unique chemical characteristics and purification problems, the compounds all have in common high polarity, a minimum range of solvents suitable for dissolution and strong retention on silica gel. Thus, alternatives were needed for separation and purification. In one case, gel permeation provided material of sufficient purity for chemical characterization from the sponge Amphimedon compressa, but final purification for full pharmacological evaluation has yet to be achieved. In the case of the tropical tree Buchenavia capitata, centrifugal countercurrent chromatography has been successfully applied to the purification of antiviral piperidino-flavanoids. The complexity of the alkaloid fraction from the tropical plant Ancistrocladus sp. required the tandem application of countercurrent chromatography and normal phase HPLC on an amino-bonded phase column, a unique application of that HPLC column previously used only in the purification of ß-carbolines from a tunicate.

Proposed Course

Our efforts in this arena continue in both the general sense of evaluating a number of approaches in every isolation project undertaken by the Natural Products Chemistry Section, as well as in focused efforts on specific targets or with specific methodology. Some examples in this latter category include the following:

1. **Separation of geometric isomers.** Our work on the Garcinia/Symphonia complex of polyisoprenylated benzophenones has produced, in addition to a number of pure compounds, two pairs of novel geometric isomers, compounds which differ only in the location of or substitution about a carbon-carbon double bond. Such mixtures comprise one of the most challenging separation problems possible. In this case, the challenge brought on by the minimal difference between the compounds is exacerbated by the tendency of these compounds to undergo acid catalyzed rearrangements. Thus, the classic approach, silver nitrate impregnated silica, is inappropriate. An alternative we are exploring is the use of a ß-cyclodextrin HPLC column. A great diversity of structure types have been separated on this packing, but very little work has been done on the preparative separation of natural products. The work reported, however, does indicate that such subtle changes in structure can be sufficient to result in resolution.

2. **Expanded application of the amino bonded phase.** This packing is very underutilized in natural products chemistry, but, as was noted earlier, it has provided some high resolution separations among several alkaloid types. We are interested in expanding the applicability of this seemingly versatile material. The most evident drawback of this bonded phase is the chemical reactivity of the amino group; carbonyl compounds are very poor candidates for successful separation and recovery.

3. **Expanded application of countercurrent chromatography.** As indicated earlier, the bulk of published work deals with polar metabolites. Recent

work has demonstrated that nonpolar compounds can be manipulated with biphasic, all organic solvent systems. It is our intention to explore this potential, using both ongoing and developing projects as investigative tools, as well as artificial mixtures of compounds from completed chemical analyses.

Publications

Cardellina JH II. HPLC separation of taxol and cephalomannine. J Liq Chromatogr 1991;14:659-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07302-01 DDRD

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Development of Chemical Characterization/Dereplication Strategies

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

PI:	John Cardellina II	Suprv. Research Chemist	LDDRD NCI
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	Mark Tischler	Visiting Fellow	LDDRD NCI
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	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*: University of Canterbury, Christchurch, New Zealand (Dr. M.H.G. Munro)

LAB/BRANCH: Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION: Natural Products Chemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.2	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.)*

Protocols have been developed for the preliminary chemical evaluation and characterization of extracts identified by the biological screens. For organic extracts, a solvent-solvent partitioning scheme is utilized to provide five fractions of increasing polarity; the location of the bioactivity is used to guide the selection of an initial chromatographic approach; NMR analyses of the active fractions are used to search for preliminary clues as to chemical class of the active agent(s). For aqueous extracts, a four-stage chemical characterization protocol employing small cartridge chromatography technology produces a profile of the elution of the bioactive components(s); this profile is compared to those of standards, e.g., sulfated polysaccharides, for purposes of dereplication, chemical characterization and selection of appropriate fractionation method.

Personnel (cont'd)

Others:	Kirk Manfredi	Senior Staff Fellow	NCI LDDRD
	Michael Boyd	Laboratory Chief	NCI LDDRD
	Louis Malspeis	Suprv. Research Chemist	NCI LDDRD
	Michael Alley	Pharmacologist	NCI LDDRD

Objectives

The high capacity screens used in the DTP program to pinpoint candidates for anti-HIV and antitumor natural products have the potential to generate a substantial number of leads per year. In three years of operation, the anti-HIV screen has, in fact, fostered nearly 1000 "leads", and the antitumor screen, now recently achieving full implementation, is likewise beginning to generate a very substantial number of initial "leads". Beyond the obvious consideration of potency in selecting extracts for chemical analysis, other criteria were required to focus our attention and efforts on the most promising leads possible. These specific aims thus evolved in the formative stages of the program:

1. Development of appropriate strategies for the preliminary chemical characterization ("chemical screening") of leads emanating from the Stage I anti-HIV and antitumor screening laboratories and application of these chemical data to dereplication or selection/prioritization of priority leads for further study.

2. Collaboration within LDDRD as appropriate to compare/contrast results of parallel preliminary biological characterization and data analysis procedures for dereplication or selection/prioritization of priority leads for further study.

Methods

1. Solvent-solvent partitioning of organic extracts.

2. Chemical screen (microcolumn chromatography) analysis of aqueous extracts.

3. Literature surveys on active genera

Major Findings

Our initial approaches to the problem represented an effort to deal with all leads, whether organic or aqueous, in the same manner. The chromatographic behavior of various extracts was examined on solid phase extraction cartridges containing silica gel, diol-bonded phase, octadecylsilyl (C_{18})-bonded phase and anion exchange resin. At this point of departure, there was a sense that nonpolar compounds would behave well on silica and diol columns, while polar metabolites could be eluted from diol and C_{18} phases. The anion exchange cartridge was included in response to expectations that sulfated polysaccharides,

sulfolipids and other acidic natural products would be encountered as HIV-inhibitory agents.

This initiative was rife with problems. The organic extraction protocol used in the NCI program yields an extract rich in polar materials. Recoveries of mass and activity from silica and diol phases were abjectly low in nearly every case studied. The aqueous extracts suffered the same fate on the C_{18} bonded phase. The worst results of all issued from the anion exchange cartridges; in no case was activity recovered. A considerable amount of experimentation led to the conclusions that the problem with the C_{18} cartridges resided in the packings narrow pore size, while the silica gel support for the anion exchange resin was the cause of irreversible adsorption.

The breadth of polarity in the organic extracts led us to reconsider a simpler alternative, a solvent-solvent partitioning scheme. A four step procedure, similar to ones performed in a number of natural products laboratories was adapted to distribute crude extracts into five fractions. This approach was perceived to offer several advantages:

1. Solvent-solvent partitioning is a rapid, simple, cost effective means of reducing the mass of the active fraction.

2. Further, partitioning is an extremely mild chemical treatment of the extract; since no solid support is used, full mass recovery can be expected.

3. The extract will be segregated into subsets of material, each of which has relatively homogeneous solubility properties. Thus, activity can be concentrated in a smaller mass which is more readily soluble in solvent combinations used in gel permeation chromatography. Alternatively, a solvent partition fraction is more likely to be manageable on selected adsorption or partition media because of the narrowed polarity range.

4. The solvent partitioning scheme, performed on a 100 mg scale for chemical screening/characterization purposes, can easily be scaled up to any size for the purposes of preparative fractionation.

5. Not only can the solvent partitioning approach be scaled up, it can also be simplified by elimination of needless steps. For example, if the activity were concentrated only in the carbon tetrachloride phase, the chloroform and ethyl acetate steps could be omitted.

6. ^{1}H-NMR and TLC analyses of the active fraction(s) can offer clues to the chemotypes present, thus permitting more informed choices of chromatographic separation methodology.

This approach has worked exceptionally well. Although there is a consistent tendency for a majority of the mass to reside in the aqueous phase, activity has been distributed in different fractions in different extracts.

We have addressed the problem of dereplication thus far in two rather classical ways. For purposes of this discussion, dereplication refers to a process whereby

leads from the screening effort due to known compounds can be readily distinguished. The first approach to dereplication consists of a search of the scientific literature to determine what chemical and pharmacological information is known about a particular genus registering activity in the screen. A number of abstracting services are employed, most notably Chemical Abstracts (CAS), Medline and Science Citation Index. There is also available a growing number of databases of individual compounds and classes of compounds (Berdy-antibiotics; Faulkner, Crews, Munro - all marine natural products; and NAPRALERT - a natural products resource emphasizing plant derived compounds). These can be consulted at the preliminary stage with queries regarding the genus in question; they are also reexamined when a pure active compound is obtained to ascertain information about that compound. The goal of this literature search approach is to obtain as much information about the active organism as possible to guide and expedite the chemical analysis. Combining literature information with data obtained from the solvent partitioning and subsequent TLC/NMR analyses could rapidly narrow the focus of the investigation to a single compound type.

The second approach to dereplication consists of a directed chemical analysis, on a small scale, to associate bioactivity with a specific chemotype. The techniques commonly employed for this purpose are HPLC with diode array detection, HPLC-MS and GC-MS. This sort of approach was recently applied by our contractor to the analysis of contamination of algal collections by a blue-green alga containing aplysiatoxin/debromoaplysiatoxin and has been employed to considerable advantage in our work on the genus Garcinia.

At this point, we were still confronted with the considerable challenge posed by a burgeoning number of aqueous extracts active in the anti-HIV screen. The striking number (approximately 20%) of aqueous extracts (algal, terrestrial plant and lichen) active in the AIDS antiviral screen has prompted us to incorporate an additional dereplication concept in the protocol under development for characterization and prioritization of those aqueous extracts. This high percentage of actives certainly suggested a recurring chemotype(s); so, dereplication was very important, not so much to associate activity with previous work (although this remained a goal), but to categorize the new leads according to bioactive constituents they held in common. A second significant problem was the chemical makeup of the aqueous extracts; there has been little work by natural products chemists on actual water extractions of organisms. Past work has focused on water soluble metabolites from alcohol or alcohol-water extracts. The constitution and chromatographic behavior of our water extracts are quite different from the aqueous soluble partition phases of alcohol extracts. Macromolecules and salts tend to be abundant, while lower molecular weight and more nonpolar compounds comprise but a small portion of the total mass.

In light of these challenges, the solid phase extraction approach still appeared to offer the best yield of information gained for the effort expended, and it offered the possibility of serving simultaneously as a tool for dereplication, characterization and selection of chromatographic separation.

Proposed Course

A dramatically revamped protocol for analysis of aqueous extracts is now being evaluated in our laboratory. The silica and diol phases have been discarded,

while a size exclusion analysis (Sephadex G-25) has been added. The C_{18} narrow pore (60 Å) phase was retained, and this reverse phase analysis has been augmented by addition of a C_4 wide pore (300 Å) phase. Finally, a polystyrene based anion exchange resin has been located and introduced to solve the problem of irreversible retention encountered in the silica based systems used earlier.

Experimental Procedure

General: Two standards are used. One is crude extract straight from its repository container (3 mg in 240 µl DMSO-H_2O, 1:1). The second is crude extract which has been exposed to 0.05% TFA (trifluoracetic acid) for 1 hr -3 mg in 0.5 ml 0.05% TFA for one hr, lyophilize, add 240 µl DMSO-H_2O, 1:1. The assay laboratory dilutes all submissions initially 1:50, then 1:2 in wells.

Reversed Phase: Baker C_{18} (Cat# 76020-06) and Baker WP C_4 (Cat# 7216-06) 6 ml cartridges and the Analytichem Vac-Elute system are used. All solvents (100% MeOH, 67% MeOH, 33% MeOH and 100% H_2O) should contain 0.05% TFA.

The cartridges are first washed with 3 column volumes of methanol, 3 column volumes of 67% methanol in H_2O and 3 column volumes of 100% H_2O. Gentle suction is used to help move the solvents through the cartridges.

The sample (3 mg) is placed on the column with a minimal amount of solvent (0.05% TFA in water is the best, $\leq$ 0.5 ml). The column is then eluted with 3 column volumes each of 100% H_2O, 33% MeOH, 67% MeOH and 100% MeOH. Each solvent system eluent is collected as one fraction. Therefore, there should be a total of 4 fractions per cartridge.

The fraction containing pure methanol is dried under nitrogen. The fractions containing MeOH-H_2O are dried under nitrogen until the MeOH is removed and then freeze-dried, as is the 100% H_2O sample. After drying, the samples are transferred to the test vials with 240 µl of DMSO-H_2O (1:1).

Size Exclusion: 1.0 g of Sephadex G-25 Fine (20-80 µ) packing is slurried in approximately 8.0 ml of 0.05% TFA in one of the Baker Sephadex G-25 column cartridges. The cartridge is shaken thoroughly to make certain that there are no air bubbles. The cartridge is rinsed with several column volumes of 0.05% TFA, using gravity flow (no vacuum should be applied during procedure).

An aliquot of 3 mg of extract in 200 µl 0.05% TFA is applied to the top of the column. All additions to the column must be done very carefully to minimize disturbing the bed of the packing material.

When the top of the sample solution has reached the column bed, 2.0 ml of eluant (0.05% TFA) is added, eluted and collected as fraction #1. A 750 µl volume of eluant is added and collected as fraction #2. Another 750 µl volume of eluant is added and collected as fraction #3. Finally, 5.0 ml of eluant is added and collected as fraction #4.

The eluants are freeze-dried and then re-dissolved in 240 µl of DMSO-H_2O (1:1) for testing.

Anion Exchange: Rainin SAX isolation cartridges are used. A 3 ml syringe is employed as solvent reservoir and source of flow pressure. The column cartridge, as purchased, is shipped in MeOH and should be stored in same. The procedure is sequenced as follows:

1) The column is initially rinsed with 6 ml H_2O.

2) The sample (3 mg) is applied in 0.5 ml H_2O.

3) The column is eluted with 1.5 ml H_2O. This is collected as fraction 1.

4) The column is eluted with 1.5 ml 0.025 M NH_4OAc. This is collected as fraction 2.

5) This column is eluted with 1.5 ml 0.25 M NH_4OAc. This is collected as fraction 3.

6) This column is eluted with 2.0 ml 2N HOAc. This is collected as fraction 4.

Each fraction is freeze-dried and reconstituted with 240 μl DMSO-H_2O (1:1) for testing.

This revised protocol was first tested on a series of known HIV-inhibitory compounds. This analysis was intended to validate the approach in terms of recovery of bioactivity and diversity of chemotypes amenable to the approach. Six anti-HIV standards were used in this analysis: suramin, AZT, cyclosporin, dextran sulfate, ATA polymer and oxathiin carboxanilide (NSC 615985). The results of these six trials were illustrative of the challenge of dealing with large molecules and/or highly charged species.

Low molecular weight, neutral or basic compounds have an elution profile characterized by substantial retention on Sephadex G-25 and rapid elution from the anion exchange column. Examples of this type include AZT, oxathiin carboxanilide and cyclosporin. The polarity differences are reflected in relative retention volumes on C_4 and C_{18} bonded phases; AZT elutes earlier than the others. Cyclosporin is aberrant on the C_{18} and anion columns. It appears to precipitate on the ion exchange bed, while its strong retention on C_{18} reflects the difficulty of dealing with peptides on small pore size columns.

The other three compounds are anionic species of differing molecular weight, polarity and ionic strength. Dextran sulfate is a very polar macromolecule, eluting rapidly from size exclusion and bonded phases, but it is strongly retained on the ion exchange resin. It is readily distinguished from the ATA polymer, which has lower molecular size and is well retained on C_4/C_{18}, and suramin, which shows intermediate retention on C_4/C_{18}, but binds to Sephadex G-25.

Approximately 20 water soluble extracts, representing plants, cyanobacteria and marine invertebrates have been examined with this protocol. The results are not as clearly interpretable as those of the standards. Solubility, precipitation and irreversible retention problems seem to have been magnified, but some useful

conclusions can be drawn from the results.

First and foremost, the mass applied to the cartridges must be varied to conform with differences in EC_{50} values for the crude extracts. If the EC_{50} is low, application of 3 mg of extract (predicated on a starting test dose of 250 µg/mL) would overload the cartridges and smear activity through several fractions on each cartridge. Conversely, too low an applied mass could result in failure to detect meaningful activity in the matrix. The former problem was, in fact, encountered with the standards; the various active compounds had to be applied in very low doses to avoid activity spilling over into multiple fractions.

Much remains to be done in this arena:

1. Continued "debugging" of the protocol. A significant number of the trial runs with crude extracts suffered from low or zero recovery of activity from one or more phases. Besides continued efforts to improve solubilization, alternative sources of bonded phase cartridges will be explored. One possibility is to look at non-silica based C_{18} packings; such material is available in HPLC columns and may be obtainable for this work. Cost and pore size may be detrimental factors.

2. Modification of the protocol to include other chromatographic media. A particularly intriguing possibility, in view of the increasing number of alkaloids encountered in this program, would be a cation exchange resin. Another option would be to replace the C_{18} narrow pore cartridge with a phenyl bonded phase or to eliminate it altogether. The argument for retaining the C_{18} phase is that it facilitates the detection of macromolecules, but it is redundant for the C_4 in most other cases. The phenyl bonded phase might show an informatively different elution pattern for certain classes of compounds; it is under-exploited in natural products research.

3. Application to prioritization of aqueous extract fractionation. This will be accomplished in two ways. First, it will serve as a dereplication tool to identify those extracts whose activity is due solely to anionic macromolecules, most probably sulfated polysaccharides, polyphenolics and acidic glycosides. Marine algae, for example, are well known commercial sources of carrageenan sulfates. The high activity rate in this group of organisms can be attributed in large measure to those constituents. However, this characterization protocol can confirm that supposition by comparison of chromatography matrix patterns. It can likewise focus our attention on those extracts, anticipated to be relatively few in the algae, which show different profiles in the protocol. Second, this chemical characterization protocol can be used to select for unique profiles that do not resemble the standards or to search for potentially desirable classes, such as novel nucleosides or lower molecular weight anionic species.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07303-01 DDRD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Anti-HIV Sulfated Polysaccharides from a Marine Sponge and a Tunicate

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

PI:	John Beutler	Cancer Expert	LDDRD NCI
Others:	John Cardellina II	Supervisory Research Chemist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION
Natural Products Chemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

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

Ultrafiltration, size exclusion chromatography, and ethanol trituration have concentrated anti-HIV activity of sponge extract in a high molecular weight fraction whose ^{13}C-NMR and elemental analyses are consistent with a sulfated 6-deoxy-polysaccharide. The same approach has led to similar results with an aqueous extract of tunicate. The trituration/^{13}C-NMR analysis approach is proving to be a useful confirmation for the chemical characterization/-dereplication protocol's indication of sulfated polysaccharides.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Objectives

1. Identification of the anti-HIV bioactive compounds in the water extract of the sponge, Reniochalina stalagmitis.

2. Identification of the anti-HIV bioactive compounds in the water extract of the tunicate Pyrosoma sp.

3. Full characterization of the active chemical species to provide a benchmark for the evolving aqueous extract characterization protocol.

Methods

It has been shown that a wide variety of sulfated polysaccharides have anti-HIV activity in an in vitro cytopathicity assay. This activity is due to an interaction with the HIV envelope in the membranes of target cells. Fucoidan, a complex sulfated polysaccharide from the alga Fucus vesiculosus, was active and synergistic with AZT versus HIV infection in vitro. No cytotoxicity was observed, even at 1000-fold increases over the protective dose. Sulfated homopolysaccharides appear to be more active than sulfated heteropolysaccharides.

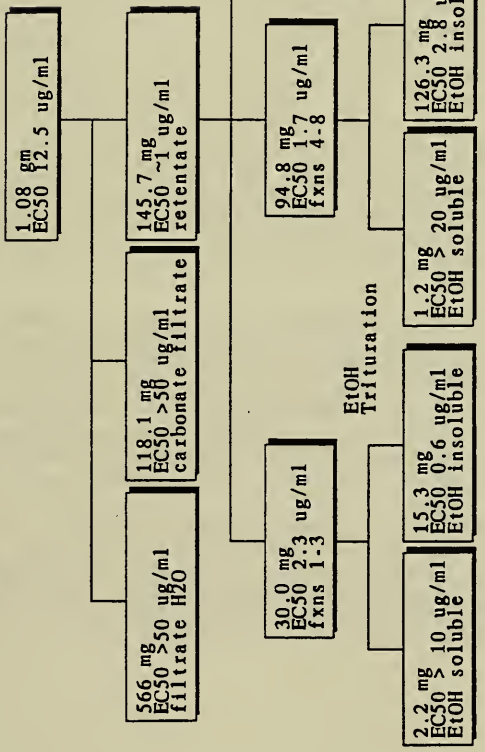
Since a large number of anti-HIV active leads have been identified in water extracts of our marine specimen collections, the characterization of these aqueous extracts assumes increasing importance. Of 377 marine invertebrate aqueous extracts tested, 84 (22%) were active. Of 254 marine plant aqueous extracts tested, 148 (58%) showed activity. While it is tempting to assume that many or most of these leads are sulfated polysaccharides, we felt that a thorough examination of several of the collections would effectively test this assumption and develop some separation strategies generally applicable to aqueous extracts. Two representative extracts were analyzed by ultrafiltration, gel filtration, high resolution size exclusion and ion exchange chromatographies. ^{13}C NMR spectroscopy was used to characterize the bioactive fractions obtained.

Major Findings

The crude aqueous extract of R. stalagmitis, an orange sponge collected in shallow waters of the Australian Great Barrier Reef, had an EC_{50} of 12 μg/ml in the HIV cytopathicity assay, with no apparent cytotoxicity in the concentration range tested. A preliminary small scale ultrafiltration (5kD) indicated that the active fraction was of high molecular weight, and this result was confirmed by a series of gel filtration experiments. The aqueous extract from Pyrosoma had an EC_{50} of 66 μg/mL and behaved similarly in the ultrafiltration experiment.

The isolation protocol used for the Reniochalina extract is outlined in Scheme 1. An essentially similiar procedure was performed in parallel with the Pyrosoma extract. The Reniochalina extract was ultrafiltered through a 5kD filter; the retentate was active in the HIV cytopathicity screen, while the filtrate was inactive. A Sephadex G-75 column was used to separate the retentate by size exclusion. All of these fractions had anti-HIV activity, and it is likely that they differ in molecular size. Trituration of these fractions in EtOH removed

Scheme 1. Fractionation of anti-HIV activity of Reniochalina stalagmitis aqueous extract.



varying amounts of non-carbohydrate material. The ethanol-soluble fractions were uniformly inactive, while the highest molecular weight ethanol-insoluble fraction had an EC_{50} of 0.6 μg/ml.

High resolution size exclusion chromatography (TosoHaas TSK-gel column, H_2O eluant) was used in an attempt to measure the molecular weight of these materials versus known standards. While the SEC column was quite useful for monitoring the crude size distribution in fractions, calibration with known standards indicated that the anionic polymers were being excluded ionically from the resin, giving misleading results. Thus, no precise estimate of the size of these materials has been made at present, but they are clearly of high molecular weight.

The three bioactive carbohydrate fractions after gel filtration, but before EtOH trituration, were examined by ^{13}C-NMR in D_2O at 70°C. The two larger fractions gave identical results, while the smallest fraction was apparently heterogeneous. Important features in the spectrum included the absence of carbonyl peaks, a methyl resonance at 16 ppm, five anomeric carbons at 99 ppm, and numerous CHOH resonances between 60 and 80 ppm. Taken together, these indicated a polymer with considerable 6-deoxy-sugar content, no N-acetyl or O-acetyl functionality, and absence of the anhydrogalactose unit found in the agarose series of polysaccharides. It should be noted that the bioactivity of the samples was undiminished after 14 hrs at 70°C, further indication that the bioactivity was due to thermostable polysaccharides.

Combustion analysis of the active fraction from Reniochalina showed the presence of 4.40% sulfur, as well as 7.49% nitrogen, 38.73% carbon, and 6.38% hydrogen. These values are consistent with a sulfated polysaccharide. The high content of nitrogen is unprecedented, and cannot be attributed to an N-acetyl functionality as seen in the chondroitin sulfates, since the ^{13}C-NMR data clearly excludes acetylated functionalities. The Pyrosoma active fraction had a similar sulfur analysis (4.32%). The levels of sulfur seen are consistent with one sulfate group for every four carbohydrate residues, while one nitrogen is indicated for every six carbons.

Several polysaccharide standards were obtained and examined for anti-HIV activity. Of these, fucoidan was highly bioactive, with an EC_{50} of 0.55 μg/ml, and heparin sulfate was weakly active at 10 μg/ml. Chondroitin sulfates A and B, arabic acid, alginic acid, and dextran were all inactive at 10 μg/ml. ^{13}C-NMR spectra were also obtained on these materials for comparison. While the fucoidan NMR spectrum is similar in some ways to the Reniochalina active fraction, it is more complex and contains a definite carbonyl resonance at 181 ppm, and an acetyl methyl at 23 ppm, neither of which are seen in our material.

Based on these comparisons, the Reniochalina polysaccharides appear to be sulfated fucans, similar to but distinct from fucoidan, a known active material. The differences include a high nitrogen content and a lesser degree of branching than fucoidan. The Pyrosoma active material is similar but considerably less abundant and homogeneous than that from Reniochalina.

Proposed Course

In future work, several lines of inquiry will be pursued to characterize these active materials more completely. We plan to use IR spectroscopy to estimate the degree of sulfation rapidly and conveniently, since combustion analysis is slow and consumes a large mass of material. This technique has been used for carrageenan and agar samples; it involves comparison of the IR absorbance of films at two absorbance bands, one (2920 cm^{-1}, C-H) representing total sugar content, and the other (1250 cm^{-1}) representing total sulfate.

A more accurate measurement of molecular weight would be desirable, perhaps by laser light-scattering techniques. Capillary electrophoresis may also be a viable means of separating and detecting sulfated saccharides.

The development of a gentle hydrolysis protocol which would not remove sulfate groups would lead to information on the particular sugars involved in the polysaccharide. Sulfate groups can be hydrolytically removed from sulfated polysaccharides under conditions (0.25N HCl, 100°, several hours) used to hydrolyze the glycoside linkages. Milder conditions have been found for fucoidan which allow the recovery of the sulfated monosaccharide (0.05M H_2SO_4, 100°, 1 hr), and should be applicable to our polysaccharides. The location of nitrogen in this polysaccharide is a key structural feature yet to be established.

We are also attempting to develop a dextran sulfate binding assay in collaboration with Dr. McMahon, so that a confirmation of sulfated polysaccharide content in aqueous extracts may be readily obtained in conjunction with the aqueous protocol.

Lymphocytes have been found to express cell surface binding sites for sulfated polysaccharides such as fucoidan and dextran sulfate. These binding sites may be associated with cell-surface receptors. Since the observed specificity of these binding sites appears to parallel the activity versus HIV, it appears that a competition binding assay for sulfated polysaccharides would be an informative tool for evaluation of HIV-active aqueous crude extracts.

The development phase of such an assay must demonstrate that the binding is saturable, specific, and dose-responsive. A number of parameters of the assay must be defined, including buffer pH, temperature, tissue source and concentration, and ligand. An examination of the references cited suggests that 37°C, pH 7.3 and physiological osmolarity would be appropriate starting parameters. For a tissue source, CEM cells would be both convenient and relevant. Fluorescein (FITC) labelled dextran sulfate is commercially available and convenient to measure by fluorimetry. FITC-labelled dextran sulfate has been shown to bind in a specific manner to living CEM lymphocytes by Dr. McMahon. The fluoresence was reduced by unlabelled dextran sulfate but not by unlabelled dextran. High background fluorescence has prevented development of a quantitative assay protocol using the FITC label, however. ^{14}C-labelled dextran sulfate will be used next to determine if an isotope label gives better results. Once the protocol is demonstrated to function, more convenient formats such as binding to fixed cells or a membrane filtration assay will be developed.

Aqueous extracts which have shown a sulfated polysaccharide profile in the chemical characterization protocol will then be tested directly in this binding assay. While it is true that extracts containing dextran sulfate binding activity might also contain other bioactive compounds, the chemical characterization protocol should give indication of that. Further, this possibility could be examined by secondarily testing gel filtration fractions.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07304-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Anti-HIV Alkaloids Isolated from Buchenavia capitata

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

PI:	John Beutler	Cancer Expert	LDDRD NCI
Others:	John Cardellina II	Supervisory Research Chemist	LDDRD NCI
	James McMahon	Biologist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

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Natural Products Chemistry Section

INSTITUTE AND LOCATION

NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Bioassay-guided fractionation of the leaf extracts of Buchenavia capitata via partition chromatography on silica and high-speed countercurrent chromatography has led to the isolation of several piperidine-flavonoid alkaloids with anti-HIV activity. The lead compound, O-demethyl buchenavianine, shows activity at 100 ng/ml, while other homologs isolated thus far are less active. The compounds are being thoroughly characterized by ^{1}H and ^{13}C-NMR (including HMBC and HMQC) and high resolution mass spectrometry.

Objectives

1. Identification of the anti-HIV bioactive compounds in the organic extract of leaves of Buchenavia capitata (Combretaceae).

2. Investigation of bioactivity in the aqueous extract of the wood of the same species.

3. Re-isolation of sufficient material(s) to provide for detailed biological evaluations; collaboration in planning and support of detailed biological evaluations by the LDDRD.

Methods

The genus Buchenavia consists of about 20 species found in tropical America. A series of flavonoid alkaloids was recently isolated from Buchenavia capitata and B. macrophylla by the Gif-sur-Yvette group in France without any report of bioactivity. No chemical work had been previously reported on this genus; these are the first alkaloids isolated from the family Combretaceae, the source of the antitubulin combretastatins.

Bioassay guided fractionation will be used on a small scale to dereplicate the alkaloids, i.e., to associate the antiviral activity with those structures.

Adaptations of those chromatographic techniques will then be employed to provide sufficient quantities of the compounds for detailed biological evaluation.

Major Findings

The initial partition evaluation of the anti-HIV active organic extracts showed the total bioactivity split among the water, $CHCl_3$ and CCl_4 fractions, although the $CHCl_3$ fraction was the most potent. A base extraction scheme gave enhanced activity in the crude base fraction, so we proceeded on the hypothesis that alkaloids were responsible for the activity. The most efficient initial treatment of the crude organic extract consisted of basification of the crude extract with 30% ammonium hydroxide and methanol, followed by partition between EtOAc and water. TLC of this organic base fraction showed six spots positive to Dragendorff reagent.

Flash chromatography of the base fraction on silica with a step gradient of $CHCl_3$ and MeOH resulted in isolation of one of the most abundant and least polar alkaloids in relatively pure form. NMR studies of this material and comparison with the literature showed that the compound was O-demethylbuchenavianine [1]. It was also the most potent bioactive fraction, with an EC_{50} of 90 ng/ml.

This lead fraction and the next most active fraction were flash chromatographed on silica with hexane-acetone to give nearly pure alkaloid. Further countercurrent chromatography of this material confirmed the attribution of bioactivity to the alkaloid, since the pure alkaloid and activity coeluted. 2D-

NMR spectroscopy was utilized to confirm the structure and to revise the carbon assignments for this compound.

The more polar alkaloidal fractions had lesser bioactivity, but were pursued to obtain congeners of the lead compound for structure-activity comparisons. A silica partition system was useful in separating polar alkaloids with weak activity. The fraction eluting from silica in $CHCl_3$-MeOH (1:1) was chromatographed in the partition system $CHCl_3$-MeOH-H_2O-HOAc (180:20:1:1), yielding a weakly active alkaloid fraction.

Further chromatography by high-speed countercurrent chromatography on a Sanki apparatus nicely resolved the alkaloid from nonalkaloidal material. The parameters used were descending mode, $CHCl_3$-MeOH-0.5% aq. HCl (5:5:3), 400 rpm, 500 psi, 1.6 ml/min., cartridge volume 250 ml. This system manipulates the basicity of the alkaloid versus the nonalkaloidal components to effect separation.

N,O-didemethylbuchenavianine [2] was isolated in pure form and subjected to 2D-NMR characterization. It was less active than 1. It was interesting that removal of a methyl group at nitrogen leads to substantially lower potency. This may be explained by the observation that tertiary amines are often better transported through biological membranes than the corresponding secondary amines.

Further countercurrent work using the same solvent system led to the isolation of buchenavianine [3], also with low activity. NMR shift data and mass spectral data were consistent with the reported structure. The use of centrifugal countercurrent chromatography to purify these alkaloids is notable since all but the least polar were poorly separated on normal phase chromatography. In the reverse-phase systems attempted, they also did not chromatograph well. The ability to vary the pH in the separation provides access to changes in the partition coefficient (and thereby the retention behavior) of basic or acidic compounds. The use of CPC served as a useful foil to normal phase chromatography, where the pH adjustment is more limited. In addition, CPC was most effective in separating the more polar alkaloids, a task for which normal phase chromatography was unsuitable.

The last and most polar group of alkaloids was freed of nonalkaloidal material by countercurrent chromatography, and separation of the individual bases is currently being pursued.

A preliminary test of the aqueous bark extract for alkaloids was negative, and the distribution of activity in the aqueous dereplication protocol indicated that the active principle was anionic and polymeric. Polymeric phenolics were postulated as the active agents.

Several alkaloids remain to be purified and identified from the organic extract of B. capitata. The structure of buchenavianine [3] remains to be confirmed by 2D-NMR studies. The other more polar alkaloids will be rigorously characterized in a similar fashion when pure, and their bioactivities will then be determined.

Since the genus Buchenavia encompasses about 20 Caribbean trees, it should be possible to collect other species of this genus to examine for other alkaloids

in this class. Chemical modification of the parent structures will also be explored by simple reactions. It is clear that relatively small modifications of the lead structure have a marked effect on activity, and it appears that intramolecular hydrogen bonding plays a role in these effects. The mechanism of action of these alkaloids has yet to be determined.

Since several structurally similar alkaloids are reported in the literature from other sources, we obtained several of these analogues for testing. Rohitukine [4] was active (EC_{50}= 1.8 μg/ml), although weaker than our lead compound 1. Rohitukine has been noted by others to have immunopharmacologic properties. The synthetic dimethylaminomethylene-substituted flavonoid 5, also obtained from the NCI repository, was not active. The genus Ficus is known to make the piperidino analogs of the Buchenavia alkaloids; however, we have not yet seen activity in the many collections of Ficus screened in the NCI program. The Schummaniophyton chromone alkaloids also bear a strong resemblance to our lead compound, lacking the C ring of the flavonoid portion. Lastly, the marine product tubastraine was reported to have a structure similar to rohitukine.

Proposed Course

We intend to pursue these structure-activity comparisons further by testing crude extracts of plants known to produce related compounds, pure compounds isolated from them, structural analogs selected from the NCI repository, and semisynthetic derivatives of the pure compounds. We also intend to track the water extract activity through bioassay-guided fractionation.

Since the isolates show some cytotoxicity, they will be evaluated in the in vitro human disease oriented tumor cell panel.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07305-01 DDRD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Lutein, a Xanthophyll with Anti-HIV Activity

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

PI:	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI
Others:	John Cardellina II	Supervisory Research Chemist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION
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NIH, NCI, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 organic extract of the leaves and stems of two plants, Excoecaria guineensis and Combretum bracteatum, were active in the NCI anti-HIV assay. The extracts were also found to give positive results in a phorbol ester binding assay. Tandem application of these two assays to direct separations led to the identification of the dihydroxy carotenoid lutein as the major active compound in Combretum; lutein was also isolated from Excoecaria extracts. The behavior of lutein and several related xanthophylls in a battery of secondary screens is being investigated.

Objectives

1. Isolation, purification and identification of anti-HIV constituents from the organic extract of Combretum bracteatum.

2. Probe the relationship of activity in the PDBu binding assay to anti-HIV activity in non-phorbol ester producing plants.

Methods

1. Use anti-HIV and PDBu assays to guide fractionation and isolate/purify natural product(s) responsible for the biological activities.

2. Determine the structure of the active constituent(s) by spectroscopic and chemical means.

3. Evaluate, in detail, the biological activty of the isolates.

Major Findings

The organic extract from C. bracteatum was submitted to a liquid-liquid partition scheme. The majority of the PDBu and HIV activity was located in the carbon tetrachloride fraction. In terms of mass, this accounted for 5% of the total extract (1.6 g from 28 g). This active fraction was further separated on Sephadex LH-20 (MeOH-CH_2Cl_2, 1:1) to give a fraction (168 mg) with an EC_{50} of 4.4 μg/ml in the anti-HIV assay and 74% displacement at 10 μg/ml in the PDBu assay. This material was bright orange-yellow and appeared to decompose slowly on standing or on exposure to ambient light. Consequently, this material was kept out of light and air as much as possible. The sample was then further fractionated using centrifugal partition chromatography (CPC) to yield a brightly colored substance which showed 85% displacement (at 5 μg/ml) in the PDBu assay. This material was further purified by HPLC using reversed phase C_{18} gel. The resulting pure material had a EC_{50} value of 1.5 μg/ml in the HIV assay and showed 65% displacement of PDBu at 2 μg/ml. Spectroscopic studies showed this compound to be the known plant pigment lutein, 1. The compound proved to be identical in all respects to a commercially available sample (Sigma Chemical Co.).

It seemed remarkable that a compound as common as lutein would be active in both the XTT-HIV and PDBu assays, although the ubiquitous sulpholipids are also HIV-inhibitory.

There has been one report in the literature on the biological activity of lutein. In that report, the authors stated that lutein uncouples oxidative phosphorylation in isolated mitochondria in vitro. Whether this is the basis for the effect observed in the anti-HIV assay is yet unknown. It should be noted that lutein is toxic to the uninfected cells in concentrations above 10 μg/ml. In terms of the PDBu activity, it should not be surprising that a compound as non-polar as lutein has some effect on membrane binding. Its activity in the PDBu assay is marginal in comparison to phorbol dibutyrate (EC_{50}= 16nM, versus

3.5 μM for lutein). Whether lutein is acting at the specific site or simply perturbing the membrane near the binding site is undetermined.

Proposed Course

Beyond the obvious need to know lutein's behavior in the LDDRD's battery of secondary screens used to confirm and quantify anti-HIV activity, additional experiments are suggested by the preliminary results described above.

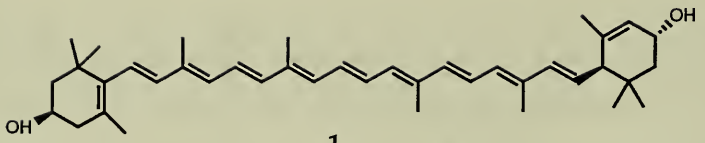
A series of related carotenoids, including ß-carotene, zeaxanthin [2], and canthaxanthin [3], has been selected for comparative evaluation in the anti-HIV and PDBu assays. Commercially available lutein will be compared side by side with the lutein isolated from C. bracteatum.

A related issue relates to the availability of lutein or its analogs, should one or more be active. All these pigments are widely distributed in the plant kingdom; if such a common metabolite were active, one should expect a very high percentage of extracts to be active as well. The most logical explanation derives from this laboratory's experience with sulfolipids. In that case, the relatively modest potency of the sulfolipids made detection of the activity in the crude extracts likely only if the sulfolipids comprised a substantial proportion of the total extract. Such was the case for some cultured cyanobacteria. A similar phenomenon may be at work in the case of lutein, since it usually occurs as a minor constituent. Support for this line of reasoning was gained by our recent tracking of some of the anti-HIV activity in extracts of Excoecaria guineensis, a member of the Euphorbiaceae, to lutein.

The further biological investigation of lutein will require a reliable and more abundant source of the compound. Our search for such a source will begin with a study of the chemical literature, to be followed by some analytical work, qualitative and quantitative, on candidate extracts.

Publications

None



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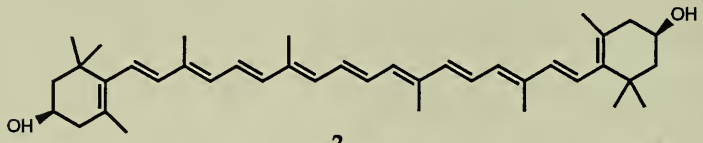

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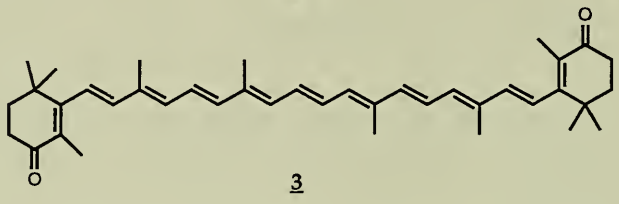

3

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07306-01 DDRD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Anti-HIV Dimeric Alkaloids from Ancistrocladus sp.

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

PI:	Kirk Manfredi	Senior Staff Fellow	LDDRD NCI
Others:	John Cardellina II	Supervisory Research Chemist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*
University of Canterbury, Department of Chemistry, Christchurch, New Zealand (Prof. J. W. Blunt); Laboratory of Analytical Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases (Dr. L. Pannell)

LAB/BRANCH
Laboratory of Drug Discovery Research and Development, DTP, DCT

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

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

The organic extract of the leaves and stems of Ancistrocladus sp. was found active in the NCI AIDS-antiviral assay. Bioassay-directed separations using centrifugal countercurrent chromatography followed by HPLC on an amino-bonded phase column resulted in the identification of a novel dimeric alkaloid. In addition to the active alkaloid, several related but inactive compounds were identified. Structure elucidation and modeling studies are nearing completion; preparative isolation methods are being developed to provide additional material for biological studies.

Objectives

1. Isolation and identification of anti-HIV constituent(s) from Ancistrocladus sp.

2. Preparative re-isolation of additional amounts of compound(s), as required, for detailed biological investigations; collaboration in planning and support of detailed biological evaluations by the LDDRD.

Methods

1. Acid-base partitioning, centrifugal partition chromatography and amino-bonded phase chromatography to purify individual antiviral alkaloids.

2. Structure elucidation, primarily by NMR and mass spectral approaches.

3. Stage II screening of the compounds by LDDRD.

Major Findings

Initially a small portion of the active organic extract from Ancistrocladus sp. was fractionated via a solvent-solvent partitioning scheme. The anti-HIV activity partitioned into the water and chloroform fractions. The chloroform solubles were then fractionated by gel permeation through Bio-Beads S-X4. Six fractions were obtained; two adjacent fractions were active. These fractions were combined and then further separated on Sephadex LH-20 to give ten fractions, one of which was active and represented about 50% of the mass of the parent fraction.

Based on NMR analysis and the previous literature on the genus, there was reason to consider alkaloids as the locus of activity. In an attempt to expedite purification, the active fraction was dissolved in dilute acid and extracted with $CHCl_3$. The active fraction was found in the acidic phase, revealing that the active material was indeed an alkaloid.

This base extract was insoluble in most organic solvents except for methanol and other lower alcohols. It was soluble in water when treated with strong acid and could be precipitated from water by treatment with base. The material also turned dark brown on exposure to air in neutral or basic solution. Since the molecule had very limited solubility in organic solvents, a centrifugal partition chromatography (CPC) was attempted. This type of separation system was thought to be ideal for this particular problem, because a number of solvent systems could be employed to overcome the solubility limitations that we had encountered. After some experimentation, a solvent system of MeOH-$CHCl_3$-0.5% HBr in H_2O (5:5:3) was selected. Dissolving the sample in a mixture of two phases and running the system with the lower phase as the mobile phase produced a large broad peak after several column volumes had passed through the cartridges. Reversal of the mobile phase to the aqueous layer gave a number of other small

peaks. The anti-HIV assay indicated that all the activity was in the large broad peak.

Analysis of the ^{1}H-NMR spectrum of the active substance suggested that the compound(s) was related to the known alkaloids from the genus. The four high field methyl doublets between 1.7-1.2 ppm comprised the same type of pattern that had been previously reported. Also characteristic of the known compounds were the two methyl singlets (2.25-2.35 ppm) and two methoxyl groups at 4.05 ppm. The down-field methines at 4.6 and 3.6 ppm also agreed with previous reports. There were three methylene protons between 2.0 and 2.8 ppm with another signal apparently under the methyl groups at 2.25 ppm. The aromatic region of the spectrum indicated seven signals, all of which appeared to be singlets. This represented an apparent deviation from all the previously known compounds. In all the known examples there were at least two adjacent aromatic protons. Since seven aromatic signals were also too many to be consistent with the known molecules, it was reasonable to assume that the material was probably a mixture of two or more related compounds.

To evaluate the purity of the sample, a number of bonded-phase, thin-layer chromatography systems were employed. Through the use of an amino-propyl bonded phase, eluted with $CHCl_3$-0.075% $(NH_4)_2CO_3$ in MeOH (85:15), the sample was shown to be a mixture of at least two related isomers. Larger-scale isolation was accomplished by means of HPLC, using the same solvent system with a preparative HPLC amino column.

From the spectrum it was clear that there were four aromatic singlets, a clear deviation from the known alkaloids. ^{13}C NMR analysis revealed twenty-three resonances. There were three methyl signals, one methoxyl group, one methylene (35.5 ppm) and two methine carbons on heteroatoms (45 and 49 ppm); the remaining sixteen carbons were found between 100 and 158 ppm, indicating that they were all sp^2 carbons. There was no carbonyl in the infrared or ^{13}C NMR spectra. The UV showed absorbances at 230, 262, 287, 312, 331 and 334 nm (log ϵ 4.8, 4.5, 4.2, 4.2, 4.2, 4.2). An initial mass spectral analysis failed to give an interpretable spectrum, but ^{254}californium desorption time of flight (TOF) mass spectrometry indicated a molecular weight around 750. Through the use of positive ion Fast Atom Bombardment (FAB) mass spectrometry we obtained a pseudomolecular ion at m/z 757. Accurate mass measurements of that MH+ ion established a molecular formula of $C_{46}H_{48}N_2O_8$.

Since the molecular weight indicated that there were forty-six carbons, it was obvious from the ^{13}C NMR data that the peaks observed (twenty-three signals) must each represent two carbons. Based on the literature precedents and extensive HMBC and HMQC NMR experiments, the gross dimeric structure shown below has been proposed for dimer A.

Through a series of difference nOe and NOESY experiments we have been able to determine the relative stereochemistry between the two rings, as illustrated below. The arrows represent substantial nOe effects between the protons indicated. The relative stereochemistry at C-1 and C-3 could also be determined from the nOe experiments and coupling constant analysis. The proton at 2.04 ppm was coupled to the proton at 3.58 ppm by 11.6 Hz. This indicated that these protons were pseudoaxial to each other. Likewise the coupling constant between

the protons at 2.62 and 3.58 ppm was 4.7 Hz, indicating an equatorial-axial orientation. When the methyl group at 1.6 ppm was irradiated, there was a large nOe on the methine signal at 3.58 ppm, indicating that there was a 1-3 diaxial interaction between the two spin systems. When the proton at 2.04 ppm was irradiated, large nOe's were observed for the protons at 6.65 and 2.62 ppm. When the proton at 2.62 was irradiated, nOe enhancements were observed at 7.21, 2.04, and 3.58 ppm. When the complementary nOe irradiation was conducted on the proton at 7.21 ppm, the largest nOe enhancement was observed for the proton at 2.62. Irradiation of the 6.65 signal showed an nOe for the proton at δ2.04 and one for the methyl group at δ2.28. These same observations were corroborated in a two dimensional NOESY experiment.

We have been unsuccessful in synthesizing or isolating crystalline derivatives for X-ray analysis. Therefore, we have thus far been unable to determine the absolute stereochemistry. The related compounds in the literature do not show consistent stereochemistry about C-1 and C-3; so, the literature has been of limited value in that regard.

The second compound isolated from the HPLC separation was closely related to dimer A. The ^{13}C NMR spectra had 46 carbons, rather than the 23 in dimer A, but the UV, IR and MS data of this compound were identical to those of dimer A. A cursory spectral comparison of the two isolates suggested that they might be atropisomers, but examination of the ^{1}H-NMR spectrum disclosed only three of the expected four signals for the two methylene groups in the tetrahydroisoquinoline rings (δ2.79, 2.55 and 2.15). However, integration revealed seven protons in the aromatic methyl signals at δ2.35. Conversion to the free base provided a ^{1}H-NMR spectrum that revealed all four methylene signals. The geminal pairs were signals at δ2.15/2.02 and 2.20/1.80 in the free base. Signals for the nitrogen bearing methine vicinal to each methylene overlapped at 3.68 ppm. Somewhat unusual was the inverse relationship between chemical shift and relative stereochemistry in the methylenes; in one case, the lowest field proton (δ2.15) was pseudoaxial to the vicinal methine, while the opposite relationship was observed in the second methylene.

When a difference nOe experiment was performed on dimer B, effects very similar to those in dimer A were seen. Irradiation of the signal at 2.15 ppm (free base form) resulted in a large nOe at 7.25 ppm; as expected, then, irradiation of the signal at 2.02 ppm showed an nOe to the proton at 6.85 ppm. However, in the other methylene, it was the proton (δ2.20) cis to the nitrogen bearing methine which had an nOe relationship to the most deshielded aromatic proton (δ7.3), while its geminal partner exerted an nOe on an aromatic proton at δ6.87. What was apparent from these experiments is that each tetrahydroisoquinoline ring had a different rotational relationship with the connected naphthalene ring. The proposed structure for dimer B.

Molecular mechanics (MM2) calculations on these two systems indicated that the barrier to rotation about the C5-C8′ bond is around 81 Kj/mol. This would indicate that free rotation about that bond does not readily occur. Rotation about the C6-C6′ bond between the two monomeric subunits is around 32 Kj/mol, in the range that would allow for free rotation about the bond near room temperature. Hence, there is no isolable pair of isomers resulting from conformational changes about that bond.

Preliminary biological studies on the compounds indicate that both isomers (A and B) behave identically in the anti-HIV assay, with an EC_{50} of ~ 25 μg/ml. At relatively high concentrations, they also appear to inhibit the production of reverse transcriptase.

Isolation and purification of additional quantities of the compounds is underway to provide material for more detailed biological investigations by other LDDRD investigators. We currently have crude material that should provide around 1.5g of the dimers. To improve the isolation method we have developed a large scale protocol which initially involves a classical alkaloid extract followed by a large scale centrifugal countercurrent isolation using a Sanki CPC. The CPC will yield a pure mixture of dimers that can be resolved via large scale HPLC on bonded phase aminopropyl columns.

Proposed Course

Determination of absolute stereochemistry. We have yet to be able to make a stable derivative of the dimers suitable for X-ray difractometry; hence, the absolute stereochemistry remains unknown. Thus, further work still needs to be done to determine the absolute stereochemistry. All attempts at derivatization have failed to yield isolable products. Chemical degradation is a possibility, as is aromatization of the isoquinoline system, but the latter approach would destroy the sp^3 chiral centers. These both possess potential problems as the target compounds themselves are expected to be quite unstable.

Rotational barriers. There would seem to be a relationship between conformation and pH in this system. Experiments need to be undertaken to account for the interconvertibility of the dimers. A careful measurement of equilibrium and pH might be achieved by looking at the NMR spectra of the compounds in various buffers to determine when interconversion occurs. Based on these results, we should be able to define the chemistry that occurs during interconversion.

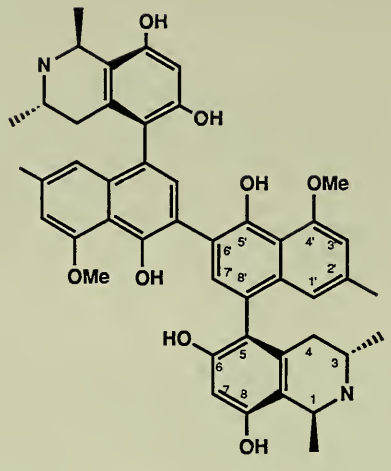
Examination of other Ancistrocladus species. The extracts of A. tectorius and A. letsutsi, already on hand, will be carefully examined for these or other dimeric alkaloids. There is currently no indication that any of the monomeric alkaloids is active. The separation strategy used effectively in this work can be applied or adapted to the other extracts.

Publications

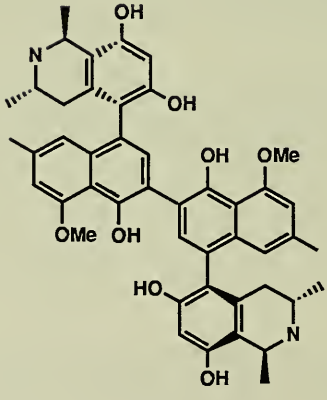
None

Patents

Boyd MR, Cardellina JH, Manfredi KP, Gulakowski RJ, McMahon JB, Blunt JH, Pannell LK, Cragg GM. US Patent 07,684,197: Michellamines Useful as Antiviral Agents, Composition and Method of Treatment, April 12, 1991.



DIMER A



DIMER B

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07307-01 DDRD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

AIDS Antiviral Plant Diterpenes

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

PI:	Kirk Gustafson	Senior Staff Fellow	LDDRD NCI
Others:	John Cardellina II	Supervisory Research Chemist	LDDRD NCI
	Michael Boyd	Laboratory Chief	LDDRD NCI

COOPERATING UNITS *(if any)*

University of Canterbury, Department of Chemistry, Christchurch, New Zealand (Prof. M.H.G. Munro, Prof. J. W. Blunt); Cornell University, Ithaca, New York (Prof. J. Clardy); Brigham Young University, Provo, Utah (Prof. P. Cox)

LAB/BRANCH

Laboratory of Drug Discovery Research and Development, DTP, DCT

SECTION

Natural Products Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, FCRDC, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Atisane and kaurane diterpenes have been isolated from extracts of two trees from tropical rainforests, Homalanthus acuminatus and Chrysobalanus icaco. One compound from each skeletal type was marginally active; three of the isolates are novel compounds. Some insight has been gained regarding structural features important for the bioactivity. The isolates have been used as templates for installing and testing a variety of 2D-NMR experiments.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Objectives

1. Bioassay guided isolation and purification of naturally occurring anti-HIV agents from extracts of Homalanthus acuminatus and Chrysobalanus icaco.

2. Spectrochemical characterization and complete structural identification of the AIDS antiviral constituents of these extracts. Special emphasis is placed on the initial implementation and operation of advanced high field NMR experiments for detailing structural elements of the purified compounds.

3. Preparative isolation and purification of additional amounts of these compounds, as required, for detailed biological evaluation by appropriate LDDRD staff and other collaborators.

Methods

All of the natural products extracts which are subjected to chemical investigation have been assigned to us on the basis of anti-HIV activity in the NCI screen. Fractionation procedures, therefore, are chosen to concentrate the active compounds rapidly while minimizing the chances of chemical decomposition of extract constituents. After each separation step, all fractions are monitored for the HIV inhibitory effects observed in the crude extract. Bioassay of all fractions is routinely performed in the same large scale screen used to test crude extracts. Through a careful analysis of mass recovery and changes in specific activity, a separation can be tailored to maximize the recovery of the active principles. All chemical studies are continually focused on the isolation and identification of the active compounds. Compounds from active fractions are rigorously purified before their activity is assessed in further detail by other LDDRD investigators.

During the course of the isolation and identification of the metabolites from H. acuminatus and C. icaco, a 500 MHz NMR spectrometer was installed in the LDDRD. Subsequently, an indirect detection probe was also acquired. Structural studies on the atisane and kaurane diterpenes continue to provide an opportunity to implement and utilize advanced NMR experimentation. The ability to perform proton detected heteronuclear correlation experiments (HMQC and HMBC) has allowed complete carbon and proton assignments on greatly reduced sample sizes. Additional NMR techniques which we now can use in an essentially routine mode include difference nOe, NOESY, double quantum COSY and TOCSY experiments.

Major Findings

Our initial chemical and biological analyses indicated that in addition to prostratin [1], extracts of H. acuminatus contained other anti-HIV metabolites unrelated to phorbol esters. Fractionation of the crude extract proceeded via solvent partitioning, vacuum liquid chromatography on silica, gel permeation through Sephadex LH-20 and preparative HPLC on C_{18} packing. Anti-HIV activity was traced to a fraction which contained a mixture of oxygenated diterpenes. Final resolution and purification of the individual components of this mixture were achieved during the purification of prostratin by normal phase HPLC on

silica gel. Four pure diterpenoids, two with the atisane carbon skeleton and two with the kaurane skeleton, were isolated. The structures of compounds **2-5** have been proposed on the basis of extensive spectroscopic and chemical characterization.

Compound **2** was an optically active, white crystalline solid with a molecular formula of $C_{20}H_{28}O_3$, as established by electron impact high resolution mass spectrometry (EI-HRMS). Infrared bands at 3491, 1713 and 1667 cm^{-1} were indicative of hydroxyl, carbonyl and olefinic functionalities. The presence of a secondary hydroxyl group, two saturated ketones and an exocyclic methylene could be discerned by characteristic ^1H and ^{13}C NMR resonances. The olefin and two ketone carbonyls accounted for three of the seven degrees of unsaturation required by the molecular formula; therefore, compound **2** was tetracyclic. The ^1H NMR spectrum also revealed three methyl singlets and two isolated pairs of methylene protons, one set α to a carbonyl and the other set allylic to the vinyl protons. Detailed ^1H-^1H COSY analysis, in conjunction with one-bond ^1H-^{13}C heteronuclear correlation experiments, established the presence of six proton ($-CH-CH_2-CH-CH_2-$) and five proton ($-CH-CH_2-CH_2-$) spin systems. These spectral features, along with recent reports describing the occurrence of atisane diterpenes in the taxonomically related genus Euphorbia, suggested that compound **2** was an atisane derived diterpene. Extensive long range (2-3 bond) heteronuclear correlations were used to establish the functional group substitution about the atisane carbon skeleton. Key correlations included those between the H-1 protons and C-2,3,5,10,20, between the H-17 protons and C-12,15 and between C-8 and the H-6,7,9,13,15 protons. Attempts to establish the complete relative stereochemistry through proton coupling constant analysis and nOe enhancement techniques led to some ambiguities. In order to confirm the structure and define the relative stereochemistry throughout the molecule, we have collaborated with Dr. Jon Clardy (Cornell University) on the x-ray crystallographic analysis of compound **2**. While the absolute stereochemistry of **2** was not defined, the ent-atisane configuration is consistent with that of related atisane diterpenes from Euphorbia species.

Compound **3** showed NMR resonances in $CDCl_3$ that were virtually identical to those reported for ent-16S, 17-dihydroxyatisan-3-one. Our complete ^{13}C and ^1H NMR assignments, using COSY and ^1H-^{13}C correlations made in C_6D_6, were fully consistent with structure **3**. A single crystal X-ray structure determination of the 17-p-bromobenzoate derivative of **3** had confirmed the absolute configuration to be that of an ent-atisane. The co-occurrence of compounds **2** and **3** supported the proposal that compound **2** is also an ent-atisane derivative.

The molecular formula of compound **4**, $C_{20}H_{32}O_4$ by HRMS, suggested that it was also a diterpene. Infrared absorbances at 3416 and 1713 cm^{-1} were appropriate for hydroxyl and ketone groups. The ^{13}C NMR spectrum contained resonances which closely corresponded to those observed for the A-ring carbons and methyl substituents of compound **2**. Resonances for additional primary and tertiary hydroxyl groups were present and all remaining signals were for sp^3 aliphatic carbons. This suggested that the exocyclic methylene group observed in compound **2** was oxidized to a vicinol diol in compound **4**. Distinct five proton ($-CH-CH_2-CH_2-$) and eight proton ($-CH-CH_2-CH_2-CH-CH_2-$) spin systems were distinguished by COSY analysis and proton detected heteronuclear correlation experiments (HMQC, HMBC). These features could best be explained if compound **4** had a kaurane

skeleton. Important two and three bond ^{1}H-^{13}C correlations supporting this conclusion included those between the H-1 protons and C-2,3,5,10,20, between H-9 and C-1,8,10,12,14,20, between the H-14 protons and C-16 and between the H-15 protons and C-17. Diaxial nOe enhancements between H-5 and H-3 confirmed that the C-3 hydroxyl was equatorial and thus C-3 has an S configuration. The relative stereochemistry at C-9 was defined by a strong nOe interaction between H-9 and H-1β while an nOe enhancement between H-9 and H-15β indicated that the C-15/C-16 bridge was linked to C-8 and C-13 in a β-configuration. The orientation of the hydroxyl group at C-16 was revealed to be α by nOe enhancements between the H-17 protons and H-13, H-14β and H-15α. Therefore, the stereochemistry at C-16 is S. Assignments of the α and β protons on C-14 and C-15 were aided by W coupling observed between H-14α and H-15β in the ^{1}H NMR spectrum. All nOe interactions and ^{1}H-^{13}C correlations were fully consistent with the proposed new kaurane structure **4**. The large negative optical rotation of **4** suggests that it has the ent-kaurane configuration.

Compound **5** analyzed for $C_{20}H_{32}O_3$ by HRMS and showed ^{13}C NMR signals which closely matched the reported values for **6**, ent-16R, 17-dihydroxykauran-3-one. However, significant chemical shift differences observed for C-16 and C-17 of **5** relative to the corresponding C-16 and C-17 positions in **6** suggested that the C-17 hydroxy methylene of **5** was α and C-16 had an S configuration. This assignment was further supported by shifts in the ^{1}H NMR resonances of the H-17 protons in **5** relative to the H-17 protons in **6**. nOe enhancements between the H-17 protons and the H-15α proton in **5** provide further evidence for S stereochemistry at C-16. Extensive analysis of COSY and ^{1}H-^{13}C correlation experiments clearly defined the kaurane skeleton. Key long range correlations included those between C-16 and H-14α, between C-17 and H-15α, between C-4 and H_3-18, H_3-19 and between H_3-20 and C-1,9,10. All nOe interactions, particularly those between H-9 and H-5, H-11β, H-15β, supported the relative stereochemistry proposed for **5**. In analogy with the other diterpenes of this series, the large negative optical rotation of **5** suggests that it has the ent-kaurane configuration.

Chrysobalanus icaco

The organic extract of Chrysobalanus icaco also showed an inhibitory effect on in vitro HIV viral infection in the NCI screen. The extract was fractionated by sequential application of gradient elution flash chromatography on C_{18} and diol packings. Anti-HIV activity of all fractions was assessed after each separation step. Concentration of the active fractions from the diol column provided a highly crystalline material which, upon re-crystallization from hexane/Et_2O, gave the major component **7**. Repeated C_{18} HPLC of the mother liquors yielded **8** as a trace constituent.

The structure of **7** was established as ent-15-oxokaur-16-en-19-oic acid by comparison of its spectral data, especially ^{1}H and ^{13}C NMR signals, to published values. Further confirmation was obtained by methylation with CH_2N_2 to give the methyl ester **9**, whose spectral data also correlated well with literature values.

The minor metabolite **8** analyzed for $C_{21}H_{30}O_4$ by HRMS and had a 3H singlet in the ^{1}H NMR indicative of a methyl ester. While the ^{1}H and ^{13}C NMR spectra of **8** were very similar to those of **7**, characteristic NMR signals revealed an additional secondary alcohol in **8**. NMR studies, including difference nOe and heteronuclear

correlation experiments, clearly demonstrated that 8 was an 11-hydroxy methyl ester derivative of the major diterpene acid 7. Enhancements (nOe) of the H_3-20 angular methyl and both H-12 protons following irradiation of the H-11 proton established the β orientation of the 11-hydroxyl group. Additional nOe interactions between the H_3-20 methyl and the H-14α proton defined the five membered ring junction as β.

This is the first time compound 8 has been isolated as a natural product; however, the corresponding hydroxy acid is known and 8 has reportedly been prepared by esterification. The spectral characteristics of 8 were only briefly described and the ^1H NMR data that were reported do not match the data that we recorded. No ^{13}C NMR data was previously provided for 8. While the reason for the discrepancies is not clear, we are confident that the data we report are fully consistent with 8.

Anti-HIV screening of compounds 2-5 and 7-9 in the standard assay revealed some interesting structure-activity comparisons. At a concentration of 6 μg/ml, compound 2 provided a maximum of 50% protection to HIV-infected cells yet it was non-cytotoxic to uninfected control cells. However, at $\geq$12 μg/ml, marked cytotoxic effects were observed in both control and HIV-infected cultures. A similar but somewhat more potent activity profile was seen with compound 7, which was non-cytotoxic and approximately 50% protective at 0.5 μg/ml but cytotoxic at $\geq$2 μg/ml. Compounds 3-5 and 8-9 did not show anti-HIV activity, but were cytotoxic at concentrations ranging from 2-50 μg/ml. It should be noted that rather subtle structural changes to these atisane and kaurane diterpenes have significant effects on their activity. The naturally occurring acid 7 was active while the related hydroxy methyl ester 8 was inactive. In a preliminary effort to address the structure/activity relationship, compound 9, the methyl ester derivative of 7, was tested and found to be inactive. While it is not yet possible to define the structural features of atisane and kaurane diterpenes which produce anti-HIV activity, it is interesting to point out that compounds 2 and 7 both have oxygenated A-rings and a $\Delta^{16,17}$ exocyclic methylene group. The relevance of the exocyclic methylene is suggested by the inactivity of 4, which has an A-ring identical to 2. Blocking the carboxyl OH group of 7 as an ester eliminates the activity; thus, it seems some sort of hydroxyl group on the A-ring is required for activity. Compounds 2 and 7 provide only a modest level of protection from HIV infection and there is a rather narrow concentration difference between the maximum activity and the onset of cytotoxicity.

Proposed Course

1. An ongoing focus is the development of new NMR capabilities. Currently, progress is being made in implementing INADEQUATE and HMQC-TOCSY pulse sequences for routine use. Since complete NMR assignments for these compounds are now in hand, these diterpenes provide excellent model compounds for establishing proper experimental parameters and refining our techniques for data interpretation in new experiments.

2. Structurally related analogs of the active compounds from H. acuminatus and C. icaco which are available in the Developmental Therapeutics Program

chemical repository will also be tested as part of a preliminary structure/activity study.

3. If it is deemed appropriate, based on biological activity data, chemical modifications of the active natural products or analogs may be initiated. This effort will seek to define further the structural features of the molecules which are responsible for the activity and to identify the best potential drug leads within this structural class of compounds.

Publications

Gustafson KR, Cardellina JH II, McMahon JB, Gulakowski RJ, Cragg GM, Boyd MR, Ishitoya J, Szallasi Z, Lewin NE, Blumberg PM, Weislow OS, Beutler JA, Cox PA. A non-promoting phorbol from the Samoan medicinal plant, Homalanthus acuminatus, inhibits cell killing by HIV-1. J Med Chem 1991;in press.

Gustafson KR, Munro MHG, Blunt JW, Cardellina JH II, McMahon JB, Gulakowski RJ, Boyd MR: HIV inhibitory natural products. 3. Diterpenes from Homalanthus acuminatus and Chrysobalanus icaco. Tetrahedron 1991;in press.

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR PHARMACOLOGY
DEVELOPMENTAL THERAPEUTICS PROGRAM
DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The major goal of the Laboratory of Molecular Pharmacology is to carry out investigations in molecular and cellular biology that are likely to be useful in the development of new drugs or of strategies for the selective killing of tumor cells.

DNA Topoiosmerases as Targets of Anticancer Drug Action (Y. Pommier and K.W. Kohn)

Several important anticancer drugs (e.g., doxorubicin, etoposides (VP-16 and VM-26), amsacrine (m-AMSA), ellipticine, mitoxantrone and camptothecin) have a characteristic action on DNA topoisomerases. Camptothecin acts on topoisomerase I while the other aforementioned drugs act on topoisomerase II. All of these drugs, as well as many of their relatives, form stabilized complexes in which DNA strands are cleaved and the topoisomerase is covalently linked to one terminus of each strand break. These cleavage complexes are similar to intermediates in the normal DNA strand-passing process of the enzymes, but in the presence of drug the lifetimes of the complexes are greatly prolonged. The persistence of the drug-stabilized complexes appears to be a major factor in cytotoxicity. The expression of cytotoxicity however is also dependent upon the activity of DNA or RNA polymerases which may interact with the complexes to produce potentially lethal lesion. These lesions, if not repaired in time, would be likely to kill the cell. Our previous work has contributed evidence in support of this picture.

Although several chemically different classes of drugs act on topoisomerase II, the drugs are often biologically very different. When comparing drugs of different types, there is no simple quantitative correlation between topoisomerase blockage and cytotoxicity. There are many possible explanations for this biological divergence. One aspect that we are studying intensively is the DNA sequence localization of the complexes. Each class of drugs yields a distinct set of topoisomerase cleavage complexes in a DNA sequence. We have mapped the distribution of complexes produced by doxorubicin, amsacrine and etoposide in the SV40 genome. Among several regions of preferential cleavage by one or more drugs, the most prominent was in the nuclear matrix associated region. This region, where the DNA can bind to the nuclear matrix, may be functionally important, and was preferred by all of the drugs. Doxorubicin had the greatest selectively for this region, while the cleavage sites produced by the other drugs were more widely distributed. The precise DNA sequence locations of the sites within the nuclear matrix associated region however differed for each drug. Analysis of a large number of sites at the DNA sequence level disclosed marked drug-dependent preferences for particular bases immediately adjacent to the cleavage sites. This was also true for topoisomerase I cleavage sites induced by camptothecin, although the identity of the adjacent preferred bases was different. These findings suggest a model

for the structure of the complex in which a drug molecule would be stacked against a base pair on either side of one of the two possible cleavage sites of topoisomerase II (or the single possible cleavage site in the case of topoisomerase I).

Current work focusses on how drug-stabilized topoisomerase cleavage complexes lead to cell killing and on what determines the relative sensitivity or resistance of different cell lines.

Work is beginning on a project to explore a possible mechanistic relationship between topoisomerase II and retroviral integrase. The integration region and the regulatory regions in the long terminal repeats of the HIV genome will be mapped for topoisomerase cleavage sites induced by a variety of drugs.

Microtubules as Targets of Anticancer Drug Action (E. Hamel)

Microtubules are required for mitosis and for the proper functioning of the cytoskeleton. In addition to the vinca alkaloids, several newer natural products have been identified as mitotic inhibitors and are the subject of the current investigation. This project focusses on interactions with tubulin from the viewpoint of structure-activity relationships and the nature and mechanisms of the perturbations of tubulin function. The work was facilitated by collaborations with several laboratories which were the sources of a variety of structurally new compounds for the current studies.

Combretastatin, isolated from an African tree by G.R. Pettit at Arizona State University, was found to bind to tubulin at the colchicine site and to inhibit tubulin polymerization. Analogs of combretastatin A-2 and A-4, obtained from Pettit and from M. Cushman at Purdue University, are being employed in an extensive structure-activity study.

Antimitotic products derived from Dolabella auricularia, a sea animal, are being investigated, along with several derivatives synthesized in Pettit's laboratory. Dolastatin 10, the most active product from Dolabella, was found to block noncompetitively the tubulin binding of vinca alkaloids, and to perturb the interactions between tubulin and GTP. Eighteen stereoisomers were tested and have yielded a general picture of the key parts of the molecule. A dolastatin 10 resistant cell line has been isolated and appears to have an altered tubulin species which is being investigated. Another product, dolastatin 11, was found to act by an apparently novel mechanism. Instead of arresting cells in mitosis, dolastatin 11 stimulated the production of multinucleate cells.

Several analogs of colchicine are being investigated in collaboration with A. Brossi (NIDDK) who synthesized these molecules. In addition to the exploration for potentially useful therapeutic agents, an affinity label form of colchicine was made which is being used to localize the binding site of colchicine on beta-tubulin.

Several compounds of previously unknown action were referred by K. Paull for testing as possible microtubule inhibitors on the basis of characteristic cell screen cytotoxicity patterns perceived by his COMPARE program. Among the first group of compounds tested, the sponge-derived products, halichondrin B and homohalichondrin B, proved to be inhibitors of tubulin polymerization and mitosis, and noncompetitive inhibitors of the binding of vinblastine. The testing of other referred compounds is in progress.

Mechanisms of Action of Antitumor Alkylating Agents (K.W. Kohn and P.M. O'Connor)

Nitrogen mustards are still among the best available anticancer drugs, despite their indiscriminate reactivity in the cell. This class of drugs could be improved by restricting the reactivity to sites that contribute to antitumor activity. Since DNA is the most likely effective target, we investigated a series of compounds in which a nitrogen mustard moiety is connected to a DNA binding group: an acridine ring system that binds by intercalation between base pairs. The mustard and acridine moieties were connected via a straight hydrocarbon chain of variable length. The compounds were synthesized under DTP contract with the Drug Synthesis and Chemistry Branch. As expected from their DNA binding properties, the compounds were found to alkylate DNA at much lower drug concentrations compared to ordinary nitrogen mustards. Moreover, their cytotoxic potencies were greatly enhanced, consistent with the idea that the acridine moiety is carrying the mustard to the effective target. The next step, completed during the current year, was to determine whether the DNA sequence selectivity of alkylation could be optimized. The working hypothesis was that alkylation of G-rich regions in the genome would be particularly effective. We found that the DNA sequence selectivity of the acridine mustard series varies with the length of the connecting chain, and that the optimum chain length for selective alkylation at sequences such as GGG is 3. Another characteristic of the series was that the monofunctional alkylating forms were nearly as cytotoxic as the bifunctional forms. The monofunctional forms are interesting because they provide further restriction of the range of chemical reactions: they produced neither DNA interstrand crosslinks nor DNA-protein crosslinks. Hence the monofunctional acridine mustard with a connecting chain length of 3 is optimally restricted with respect to the range of chemical reactions that it would produce in the cell, and this compound is a potential candidate for drug development.

The monofunctional acridine mustards may have a distinctive mechanism of action, in which a single DNA alkylation and a DNA intercalation combine to produce the cytotoxic lesion, in contrast to ordinary nitrogen mustards whose major cytotoxic lesions are crosslinks. The entire series of acridine mustards, both mono and bifunctional, is being tested in the DTP cell screen.

The mechanism of interstrand crosslinking by ordinary nitrogen mustards is being studied in designed synthetic oligonucleotide duplexes. A gel electrophoretic assay was developed to compare crosslinking efficiencies of different nitrogen mustards and in different oligonucleotide duplexes. Thus the effects of drug structure and of DNA sequence environment can be

determined. A particularly interesting finding was that isophosphoramide mustard showed an unusually high efficiency of crosslinking, compared with HN2 or phosphoramide mustard (the reactive form of cyclophosphamide). The crosslink for all of these drugs was preferentially in the sequence 5'GNC3'.

Studies were initiated of the process by which DNA damage by alkylating agents leads to selective cell death. Burkitt lymphoma cell lines are being studied which differ in sensitivity to HN2, but which were previously shown to be alike with respect to the extents of production and the rates of repair of interstrand crosslinks and DNA-protein crosslinks. Following treatment with HN2, the cell lines exhibited G2 delay and corresponding changes in the cyclin/cdc2 kinase system which controls this part of the cell cycle. The objective is to determine where in the molecular cell cycle control network the cell lines may differ.

DNA Damage and Repair in Specific Regions of the Genome (V. Bohr)

The ability of cells to survive treatment with DNA-damaging drugs depends in part on their ability to repair the DNA lesions. Differences in the efficiency of DNA repair among different cell lines may be an important factor in drug sensitivity or resistance. The focus of this project is on DNA damage and repair in specific regions of the genome. Previous work demonstrated preferential repair in active genes as opposed to transcriptionally inactive regions, and provided evidence that this difference can be an important factor governing cell survival.

Current emphasis is on the development and application of assays of DNA lesions produced by chemotherapeutic agents, in particular platinum complexes and alkylating agents. Methodology is now in place for assays of interstrand crosslinks, intrastrand alkylations or platinations and for x-ray induced strand breaks in specific genomic regions. The preferential repair of cisplatin DNA adducts was demonstrated in the dihydrofolate reductase (DHFR) gene of Chinese hamster CHO cells. Nitrogen mustard was found to generate different densities of alkylation in different genomic regions, and these lesions were preferentially repaired in the active DHFR gene in CHO cells.

Cisplatin-sensitive and resistant human ovarian carcinoma cell lines were treated with cisplatin and compared with respect to the formation and removal DNA intrastrand adducts and interstrand crosslinks. Interstrand crosslinks in specific genes were removed more rapidly in the resistant cells than in the sensitive cells, whereas no difference was observed in the removal of intrastrand adducts. Since there was no difference in overall repair of interstrand crosslinks as measured by alkaline elution, the possibility of a difference in preferential gene repair is being investigated.

Cells from human DNA repair deficiency syndromes could be employed as a key to the relationships between specific genetic loci, cell survival and specific DNA repair processes. Deficient preferential repair of an active gene was found in Cockayne's syndrome and possibly in Gardiner's syndrome, but not in cells of some other genetic DNA repair diseases. The several complementation

groups of xeroderma pigmentosum are being investigated (in collaboration with J. Robbins). Studies with ultraviolet light have so far shown complex relationships between cell survival, overall genome repair and preferential gene repair. A key problem may be the multiplicity of DNA lesions which may be repaired differently and which may have different cytotoxic potential. Similar problems exist for most of the common DNA damaging drugs and will require extensive work to resolve.

The mechanism of preferential gene repair was investigated through the use of specific enzyme inhibitors. The preferential repair of pyrimidine dimers in the hamster DHFR gene was unaffected by aphidicolin, 3-aminobenzamide, hydroxyurea, etoposide, merbarone, camptothecin or beta-lapachone, although all of these compounds inhibited DNA replication and most of them inhibited overall DNA repair synthesis. The preferential repair was inhibited somewhat by caffeine, and this effect is being studied further. Although neither camptothecin nor merbarone inhibited preferential repair, the combination of the 2 drugs did inhibit. The possibility is being considered that preferential repair may require the action of a topoisomerase.

A possible relationship between DNA repair and the origin of B cell malignancies was found in studies comparing normal B lymphoblasts from DBA and BALB/C mice (collaboration with Dr. Michael Potter's laboratory). BALB/C mice produce plasma cell tumors much more readily than do DBA mice. DNA repair kinetics were compared in several specific gene regions. The only region showing a difference was in the 5' flank of the c-myc gene (where BALB/C plasmacytomas tend to have translocation breakpoints), and this region was repaired more slowly in BALB/C than in DBA lymphoblasts.

Experiments have been initiated to determine whether there is anything unusual about DNA repair in AIDS lymphoma or HIV infected cells.

Molecular Biology of Cellular Responses to DNA Damage and Other Injury (A. Fornace)

Cytotoxic chemotherapy depends upon the selective sensitivity of target cells to certain types of damage. DNA damage is a major factor in the action of many of the most effective anticancer drugs. This project seeks an understanding at the molecular biology level of the factors that determine the ability of cells to survive a given type and extent of damage. While the preceding project concentrates on DNA repair mechanisms, the current project aims at the general question of what genes are induced by DNA damage and what do these genes do. In previous work, a variety of cDNA clones were isolated based upon their induction by DNA damage produced by UV light. Some of the cloned transcripts were specific for UV, whereas others responded also to some other types of DNA damage. Several of the transcripts were found to be elevated in chemoresistant tumor cell lines.

Some of the DNA damage inducible clones were of particular interest, because their corresponding transcripts were also activated during cellular growth arrest. This was of interest because growth arrest in certain phases of the

cell cycle often follows DNA damage, and events occurring during arrest may favor ultimate survival. Two of these "growth arrest and DNA damage inducible" (*gadd*) genes were sequenced from genomic clones including the regulatory regions in their 5′ flanks (in collaboration with N. Holbrook). The *gadd45* and *gadd153* genes showed a remarkable degree of evolutionary conservation (collaboration with R. Nairn), suggesting that their products participate in interactions with other highly conserved macromolecules. The promoter regions of these genes were probed by deletion mapping and by means of CAT-reporter gene constructs. Within the promoter regions, DNA damage responsive elements were found, some of which may be recognition sequences for new and as yet unidentified regulatory factors. One such element showed sequence similarity in the two genes and will be further investigated as a candidate for involvement in the control mechanisms which govern growth arrest and/or response to DNA damage.

The *gadd45* gene was found to be strongly induced also by x-rays, and to be unique among the previously known x-ray-induced genes in that it does not work via protein kinase C. The induction was found to be diminished in cells from patients with the radiosensitivity disease, ataxia telangiectasia (collaboration with J. Robbins). Induction was undetectable in 11 of 17 human tumor cell lines examined, but was readily detected in all of 12 non-tumor lines tested (collaboration with R. Weichselbaum). This may be a clue to a metabolic abnormality existing in some tumors that may be therapeutically exploitable. The functional regulation of the *gadd45* gene is under intensive investigation.

Other stress-response genes were also studied, including some of the 15 different heat-shock genes whose cDNA clones were previously isolated. Some of these genes showed induction also by certain DNA damaging agents. One of these genes (hsp27), when overexpressed in cells containing an hsp27 expression vector, conferred resistance to DNA damage by UV light (collaboration with J. Landry). Hence this DNA damage inducible gene apparently does protect cells against DNA damage.

Deficiency of the DNA repair gene, guanine-O6-alkyltransferase (O6AT), has previously been implicated in the high sensitivity of some tumor cell lines to nitrosoureas. Improved clinical usefulness of nitrosoureas may hinge on the question of whether clinical tumors sometimes are deficient in O6AT and whether such deficiency would predict drug responsiveness. Pursuit of these questions requires practical assays for the expression and activity of O6AT in tumors. To this end, a full-length cDNA clone for O6AT was isolated and successfully used to measure O6AT mRNA. This RNA was found to be reduced at least 100-fold in nitrosurea-sensitive mer^- cells, compared with normal (mer^+) cells. In collaboration with D. Yarosh, the cDNA clone was used in a bacterial expression vector to make a large quantity of O6AT protein from which high affinity antibodies were developed.

Studies of the regulation of HIV gene expression in response to DNA damaging agents are being initiated with funds from the NIH Intramural AIDS Targeted Antiviral Program. This investigation is pertinent to the specific malignancies that are associated with AIDS. A promoter in the HIV long

terminal repeat region, which has been reported to be strongly induced by DNA damage, will be studied to compare its responses to various types of genotoxic stress with those of other DNA inducible genes. The participation of the HIV genome in the regulatory network associated with DNA damage responses will be probed by looking for changes in HIV gene expression produced by antisense constructs to some of the *gadd* genes.

Structure and Regulation of Histone and DNA Synthesis (W. Bonner and C. Hatch)

This project investigates the coordinate regulation that assures a stochiometric balance between the synthesis of DNA and the syntheses of the major histone species. The maintenance of this balance may be crucial to the survival of cells treated with DNA synthesis inhibitors, because excess histone is likely to be cytotoxic. Our isolation of the H2A.X gene is of special importance in these studies because it is the only known situation to date in which the synthesis of a histone protein from an apparently replication-dependent histone gene is uncoupled from replication in growing cells. Understanding the mechanism by which this uncoupling occurs in nature is a step towards our goal of being able to manipulate the balance between histone and DNA synthesis for therapeutic benefit.

Even though its structure is indistinguishable from those of the replication-dependent histone genes, the H2A.X gene was found to be unique in that it generates replication-independent as well as replication-dependent messages, leading to the classification of the H2A.X protein as a replication-independent histone. The H2A.X gene has been sequenced and studies are underway to determine how this uncoupling occurs. These studies involve altering sequences in the H2A.X gene and analyzing their effect on the type of transcripts in stably transfected cells. Chimeras of the H2A.X gene and an H2A.1 gene, which has a similar structure to the H2A.X gene but which yields only a replication-dependent message, are also being utilized to localize regions critical to the uncoupling. We have also made the observation that there appear to be significant differences in the proportion of the replication-independent and replication-dependent H2A.X transcripts between normal and transformed human cells.

The H2A.Z gene has been sequenced and its regulatory regions are being studied. A core promoter region was found to be localized in the 234 base pairs immediately upstream from the transcription start site. The next 160 base pairs upstream conferred silencer activity (i.e., the ability to reduce transcription). Evidence was obtained of specific regulatory proteins binding to defined nucleotide sequences within the silencer region. Silencer sequences may be important as a general mechanism for modulating the expression of genes that are not otherwise inducible or repressible.

As a working hypothesis, the balance between histone and DNA synthesis is presumed to be mediated by the concentration of histones in the cytoplasmic soluble pool. Direct tests of this hypothesis are hindered by the low concentrations of soluble histone normally present. New methodology was worked out which allowed a beginning to be made, and evidence was obtained

that soluble histones are bound in a negatively charged complex that sediments at 8-9 s. Further investigation however will require yet greater sensitivity, and other methodology involving epitope tagging of histones is being tested for feasibility.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06140-15 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters 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:	Christopher Hatch	Sr. Staff Fellow	LMPH NCI
	Concepcion Muneses	Chemist	LMPH NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

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

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

The primary objectives of this project are to elucidate how replication-dependent and replication-independent histone synthesis are interrelated and regulated with regard to the stoichiometry of the various histone subtypes in the nucleosome, and how replication-dependent histone synthesis is regulated with regard to the rate of DNA synthesis. Because of exciting finds in the study of the H2A.X gene, most of our effort this year has been applied to that study. (see Z01 CM 06192-03).

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 evidence suggesting that soluble histone in mammalian cells is bound in a negatively charged complex that sediments at 8-9s. Because histones have been so conserved during evolution, it has not been possible to prepare high affinity antibodies. We are currently testing the possibility of preparing transfected cell lines carrying genes for altered histone proteins that carry an epitope to a known monoclonal antibody. Such an approach would greatly increase our ability to isolate and characterize components bound to the soluble histone complex.

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:

Histones are synthesized in two manners, replication-dependent (RD) coordinated with DNA synthesis and replication-independent (RI) found in quiescent cells.

RD 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 histones (RI) 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 study the feasibility of epitope tagging histone proteins in mammalian cells in order to isolate components which interact with soluble histone. Related to this question is how much can the histone protein be altered in sequence and still be incorporated into chromatin.

3) To characterize histone mRNAs from quiescent cells (RI histone synthesis) and how their regulation is related to RD histone synthesis.

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 including oligonucleotide synthesis and PCR.

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 some 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. Initial biochemical characterization of soluble histone suggests that it is bound in a negatively charged complex that sediments at 8-9s. However, purification and further characterization of these particles has been hindered by their small amounts. Recently we have been testing the feasibility of epitope tagging histones as a means of using monoclonal antibody techniques with these complexes (it has not been possible to prepare monoclonal antibodies directly to histones). Such methodology has been used successfully with RNA Polymerase in yeast.

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 be expected to interfere with DNA replication, thus leading to alternative procedures with possible therapeutic benefit.

Proposed Course:

1. To characterize soluble histone and study its interaction with other components involved in histone mRNA stability.

2. To study the behavior of altered histone proteins in order to tag them with epitopes to known monoclonal antibodies.

3. To inhibit the nuclease action on histone mRNA in vitro and in vivio with oligonucleotides that mimic the 3′ histone mRNA stem-loop.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06150-10 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

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 Scientist	LMPH NCI
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	Akihiko Tanizawa	Visiting Fellow	LMPH NCI
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	Kurt W. Kohn	Lab Chief	LMPH NCI

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Laboratory of Molecular Carcinogenesis, NCI (Dr. K. H. Kraemer)

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 both the nuclear scaffold in interphase nuclei and the chromosome scaffold during mitosis. Topoisomerase II activity is required for chromosome segregation during mitosis and both enzymes are involved in DNA replication and transcription functions. Camptothecin inhibits topoisomerase I, and DNA intercalators (amsacrine, anthracyclines) and demethylepipodophyllotoxins (VP-16 & VM-26) inhibit topoisomerase II. The drugs act by stabilizing enzyme-linked DNA strand breaks, and these may be the initial cytotoxic lesions produced by the drugs. However, the breaks reverse quickly upon drug removal, indicating that other processes are involved in the sequence of events leading to cell death. This project aims to elucidate these events. We have obtained evidence that the topoisomerase I-DNA complexes induced by camptothecin kill rapidly proliferating Chinese hamster DC3F and slowly proliferating human colon cancer cells by interacting with DNA replication complexes. We have also demonstrated that topoisomerase-mediated DNA breaks are not toxic in cells which have been depleted of calcium and that protein kinases can modulate cellular sensitivity to topoisomerase inhibitors. Further, it now appears possible that topoisomerase I is regulated by signal transduction pathways including protein kinase C. We have started mapping cleavage sites in the c-myc oncogene and find that *m*-AMSA selectively inhibits topoisomerase II by stabilizing cleavage complexes in the P2 promoter, a site most often used for c-myc transcription in tumor cells. A new project has been the development of camptothecin-resistant cells. We have now obtained a highly resistant cell line and are studying its topoisomerases.

1. Study the cellular events leading to cell death upon induction of topoisomerase-linked DNA breaks by antitumor topoisomerase I and II inhibitors.

2. Investigate the cytotoxic effects of combined treatments with camptothecin and VP-16.

3. Isolate DNA topoisomerases from resistant cells and study the mechanisms of cell resistance at the topoisomerase level.

4. Investigate the relationships between DNA cleavage hot-spots, topoisomerase function and selectivity of drug action in tumor cells.

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 Mechanisms of Topoisomerase II inhibitors in Human Cancer Cells:

In order to understand the cellular events associated with cell death after the formation of topoisomerase II-DNA cleavable complexes, we compared the induction of endonucleolytic DNA fragmentation by teniposide (VM-26) in the human cell lines HT-29 and HL-60. Both cell lines showed similar loss of colony formation ability following 30 min treatment with various VM-26 concentrations even though the initial topoisomerase II-mediated DNA single-strand break frequency was higher in HL-60 cells. DNA repair studies following drug removal indicated that VM-26-induced cleavable complexes reversed rapidly and completely in HT-29 cells while in HL-60 cells, the initial lesions persisted at and above 5 uM VM-26. Secondary DNA fragmentation resembling chromatin endonucleolytic cleavage by apoptosis could be detected in HL-60 cells but not in HT-29 cells. Secondary DNA fragmentation was also induced by VM-26 in the human colon cancer cell lines COLO 320, which have *c-myc* amplification. Since HL-60 cells also have *c-myc* amplification and HT-29 do not, it is possible that *c-myc* overexpression may be involved in secondary DNA fragmentation. Finally our results indicate

heterogeneity of cell death mechanisms after exposure to the topoisomerase II inhibitor VM-26 in human cancer cell lines.

2. Cytotoxic Mechanisms of Camptothecin and 10,11-methylenedioxy-20-(RS)-camptothecin in Human Colon Carcinoma:

We have previously shown that: 1) killing of rapidly proliferating Chinese hamster cells DC3F by camptothecin (CPT) is dependent upon the stabilization of cleavage complexes in S-phase cells (Holm et al., *Cancer Res.* 49: 6365, 1989); and 2) slowly growing human colon carcinoma cells (HT-29) are as sensitive as DC3F cells to CPT and to its more potent analog, 10,11-methylenedioxycamptothecin (MDO-CPT) (O'Connor et al., Cancer Commun 1990;2:395). We have now determined by flow cytometry that only 21% of exponentially growing HT-29 cells are in S-phase and that 30 min exposure to CPT or MDO-CPT produced approximately 90% cell killing (by colony formation assay). Since protein-linked DNA breaks reversed within 30 min after drug removal it seemed possible that the drugs killed HT-29 cells outside of S-phase. This possibility was tested by studying the effects of simultaneous treatments with the DNA synthesis inhibitor, aphidicolin. Aphidicolin abolished the cytotoxicity of CPT or MDO-CPT without reducing drug-induced cleavage complexes. These results suggest that flow cytometry measurements underestimate the fraction of HT-29 that are in S-phase and confirm that the cytotoxicity of CPT and MDO-CPT requires active DNA replication. We propose that cell killing by camptothecins results from the collision of moving replication forks into drug-stabilized cleavage complexes.

3. Time-Dependent Antagonism between Camptothecin and VP-16 in Human Colon Carcinoma HT-29 cells:

The cytotoxicity of combination chemotherapy with the topoisomerase I inhibitor camptothecin (CPT) and the topoisomerase II inhibitor etoposide (VP-16) was investigated in human colon carcinoma HT-29 cells. Simultaneous treatments of HT-29 cells with CPT and VP-16 produced less than additive cytotoxicity compared to single agent administration. These results indicated an antagonistic response for combined modality treatment of topoisomerase I and II inhibitors. Sequential 30 min treatments with equitoxic doses of CPT (1 uM) and VP-16 (50 uM) showed that CPT protected HT-29 cells from VP-16-induced cytotoxicity for approximately 4 h after CPT removal. Thereafter, cytotoxicity increased progressively and became additive when VP-16 was given more than 6 to 8 h after CPT removal. Similar results were obtained when VP-16 was given prior to CPT. Measurement of drug-induced DNA single-strand break kinetics demonstrated that protection was not related to quantitative alteration of topoisomerase I- or II-mediated DNA-cleavable complex formation or reversal. Rather, protection was correlated with RNA and DNA synthesis inhibition since the kinetics of these inhibitions were superimposable with the cytotoxic response of HT-29 cells to topoisomerase inhibitors. These results are consistent with the proposal that both ongoing DNA and RNA synthesis are determinants in the cytotoxicity of topoisomerase inhibitors. Further, our results indicate that combination chemotherapy employing CPT and

VP-16 as topoisomerase inhibitors may be antagonistic when given simultaneously or within 4 h of each other.

4. Camptothecin-resistant cell lines derived from Chinese hamster DC3F cells in culture:

DC3F cells were first mutagenized with ethylmethanesulfonate (50 ug/ml for 16 h yielding 50% cell survival) and then selected for camptothecin (CPT) resistance by exposure to 1 uM CPT for either 1 or 10 days. Several clones were obtained after the 1-day exposure to CPT. The most resistant one (DC3F/C-1) showed approximately one log less cell kill than DC3F cells after 8 or 24 h exposure to 0.25 uM CPT. A more resistant cell line was obtained after the 10-day CPT exposure (DC3F/C-10). DC3F/C-10 cells were highly CPT resistant, showing no cytotoxicity in the presence of 0.1 uM CPT, while DC3F cells, the parent line exhibited less than 1% cell survival under these conditions. CPT-induced cleavable complexes were measured by alkaline elution of CPT-treated isolated nuclei. DC3F/C-1 cells formed less breaks than DC3F cells and DC3F/C-10 almost none. Therefore, resistance to CPT is probably linked to topoisomerase I alterations in these cell lines.

5. Influence of Topoisomerase Phosphorylation upon the Cytotoxicity of Topoisomerase II and I inhibitors:

We had previously reported that 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 investigated the modulating effects of trifluoroperazine upon the cytotoxicity of topoisomerase II inhibitors in these L1210 cells. Trifluoroperazine (TFP) reduced the resistance to topoisomerase II inhibitors, including VP-16, m-AMSA, and doxorubicin without potentiating the cytotoxicity of the topoisomerase II inhibitors in the sensitive (wild type) cells. This effect was not due to increased drug uptake or P-glycoprotein alterations but rather to an increase in drug-induced cleavage complex formation. TFP did not increase cleavage complexes in isolated nuclei or in nuclear extracts indicating that the potentiating effects of TFP upon cleavage complex formation was due its interaction with soluble factors, possibly protein kinases that could regulate topoisomerase II sensitivity to topoisomerase II inhibitors. We have also examined the relationship between protein kinase C and CPT sensitivity in Chinese hamster DC3F/9-OHE, and in Human colon cancer HT-29 and colo-320 cells. Within 30 min after addition of 0.1 uM TPA (to stimulate protein kinase C), CPT-induced cleavage complexes and cytotoxicity 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/90HE 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 findings with purified topoisomerases I (Pommier et al. *J Biol Chem* 1989;265:9412-94182) and implicate protein kinase C as a regulator of topoisomerase I activity in various cell lines.

6. Topoisomerase II Activity in Bloom Syndrome Cells:

Cells from patients with Bloom syndrome, a cancer-prone disorder with cutaneous photosensitivity and spontaneous chromosome breakage, exhibit an abnormally increased number of sister chromatid exchanges following treatment with 5-bromodeoxyuridine (BrdU). This effect has been postulated to be mediated by abnormal topoisomerase II activity. We used alkaline elution to measure DNA single strand breakage following prolonged exposure to BrdU. Five day exposure to BrdU produced equal numbers of alkali-labile sites in normal and Bloom syndrome fibroblasts. These breaks were not protein-associated but were produced by alkali. Treatment with topoisomerase II inhibitors induced similar frequencies of DNA single strand breaks in normal and Bloom syndrome fibroblasts. These findings imply that BrdU incorporation into cellular DNA induces alkali labile DNA lesions that are independent of topoisomerase II activity in Bloom and normal cells.

7. DNA Cleavage Sites Induced by DNA Topoisomerase II Inhibitors in the Human c-myc Gene:

The cleavage sites induced by purified mouse leukemia (L1210) topoisomerase II were mapped in the absence and presence of m-AMSA in 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 2944. Cleavage was on both strands with a 4 base pair stagger. This cleavage site was also detected in the absence of drug, and in human lung carcinoma NCI-N 417 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. Two other regions of strong cleavage sites were also induced by m-AMSA approximately 200 base pairs upstream from exon 1 and in the first intron. These results indicate that DNA topoisomerase II binds to the promoter regions of exon 1 of the c-myc gene and suggest that the stabilization of cleavage complexes at the P2 promoter site may be critical for antitumor activity.

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 I inhibitors: for camptothecins, determine the DNA replication alterations resulting from the "collision" of replication fork with cleavage complexes.

3. Identify the cellular lesions responsible for cell death after exposure to topoisomerase II inhibitors by investigating the relationship between cleavage complexes in the c-myc oncogene and gene transcription.

4. Investigate the role of protein kinases and signal transduction pathways in the cytotoxicity of topoisomerase inhibitors.

Publications:

Pommier Y, Kerrigan D, Hartman KD, Glazer RI. Phosphorylation of mammalian DNA topoisomerase I and activation by protein kinase C, J Biol Chem 1990;16:9418-22.

O'Connor PM, Kerrigan D, Bertrand R, Kohn KW, Pommier Y. 10,11-methylenedioxycamptothecin, a topoisomerase I inhibitor of increased potency: DNA damage and correlation to cytotoxicity in human colon carcinoma (HT-29) cells, Cancer Commun 1990;2:395-400.

Kamath N, Grabowski D, Ford J, Drake F, Kerrigan D, Pommier Y, Ganapathi R. Trifluoroperazine modulation of resistance to the topoisomerase II inhibitor etoposide in doxorubicin resistant L1210 murine leukemia cells, Cancer Commun 1991;3:37-44.

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Bertrand R, Kerrigan D, Sarang M, Pommier Y. Cell death induced by topoisomerase inhibitors: role of calcium in mammalian cells, Biochem Pharmacol, *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

PROJECT NUMBER: Z01 CM 06161-08 LMPH

PERIOD COVERED: October 1, 1990 to September 30, 1991

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

DNA Topoisomerases as Targets 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 Scientist	LMPH NCI
Others:	Francois Leteurte	Guest Researcher	LMPH NCI
	Bruno Poddevin	Guest Researcher	LMPH NCI
	Akihiko Tanizawa	Visiting Fellow	LMPH NCI
	Donna Kerrigan	Chemist	LMPH NCI
	Ann Orr	Microbiologist	LMPH NCI
	Richard Bertrand	Visiting Fellow	LMPH NCI

COOPERATING UNITS *(if any)*

Laboratory of Clinical Pharmacology, NCI (Dr. B. K. Sinha)

LAB/BRANCH: Laboratory of Molecular Pharmacology, DTP, DCT

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Topoisomerase inhibitors block topoisomerases II and I by stabilizing cleavage complexes that probably correspond to natural intermediates in the DNA strand passage reactions of these enzymes. Drugs of different chemical class stabilize different patterns of cleavage sites. The basis for selectivity of drug action was investigated by sequencing a large number of sites induced by inhibitors of topoisomerase I (camptothecin) and topoisomerase II (anthracyclines, *m*-AMSA, VP-16, VM-26, mitoxantrone and ellipticines). DNA sequencing was performed using purified mouse leukemia L1210 topoisomerases and ^{32}P-end labeled DNA fragments from SV40 DNA and the human MYC gene. A computer program was developed in order to analyze the statistical probability of a random occurrence at each position between positions [-20] and [+20] from the cleavage sites. For topoisomerase I in the absence of drug the strongest preference was T[-1]. Camptothecin stabilized a subset of those sites with a strong preference for those having G[+1]. For topoisomerase II in the absence of drug a core region of strong base preference was found between positions [-3] and [+7] but no single base was strongly preferred at any of these positions. For anthracyclines, A[-1] at one of the cleavage sites of a DNA double-strand break was always present. For acridines (*m*-AMSA), it was A[+1], and for epipodophyllotoxins (VP-16 and VM-26), it was C[-1]. We now propose a model in which topoisomerase inhibitors bind inside a cavity formed by the base pairs flanking the cleavage site (-1 and +1 bases) and the enzyme.
We have isolated a Chinese hamster cell line resistant to camptothecin. Since purified topoisomerase I from these cells is camptothecin-resistant, we are planning to clone the topoisomerase I gene(s) in order to determine the mutation sites. Mutation sites may correspond to amino acids that interact with camptothecin.

Professional Personnel (Continued):

Kurt W. Kohn	Lab Chief	LMPH NCI

Objectives:

1. Use purified enzymes and DNA fragments to compare drug potencies and sequence selectivity and to select the most active topoisomerase inhibitors that could be studied in cells and animal tumor models.

2. Map the DNA sequence locations of topoisomerase cleavage sites in mammalian genes and viruses.

3. Determine the molecular interactions between drugs and topoisomerase-DNA complexes.

4. Compare drug-induced DNA cleavage in free DNA and in chromatin.

5. Clone topoisomerase genes from sensitive and drug-resistant cells

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.

4. Computer analysis of base sequence preferences at topoisomerase cleavage sites.

5. Gene cloning using topoisomerase I antibodies and genetic probes for library screening.

Major Findings:

1. DNA Sequence Requirements for Topoisomerase I-Induced DNA cleavage and Inhibition by Camptothecins:

178 DNA cleavage sites induced by topoisomerase I in the absence of drug were sequenced as well as 44 sites induced in the presence of camptothecin. These sites were aligned relative to the base immediately 5′ to the cleavage sites (covalent linkage to topoisomerase I at the 3′-DNA terminus). Deviation of base distribution at a given position was determined by Chi-square analysis. In the absence of drug, a 9 base pair core region of non-random base preference was found between positions -6 and +3. The strongest bias was at position [-1] with strong preference for T. In the presence of camptothecin which enhanced cleavage at a subset of enzyme cleavage sites, another strong preference was G[+1]. In order to test a possible 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 (Jaxel et al., *Nucleic Acids Res.*, 16: 11157, 1988). 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.

2. DNA Sequence Requirements for Topoisomerase II-Induced DNA cleavage and Inhibition by Anthracyclines:

We have shown previously that the DNA cleavage patterns of anthracyclines were similar and that strong cleavage sites were concentrated in the SV40 nuclear matrix attachment region. Anthracyclines differed from each other by their relative potency rather than by their DNA sequence selectivity of cleavage (Capranico et al., *Biochemistry 1990;*29:562). We have now investigated the basis for base selectivity. Ninety eight cleavage sites induced by topoisomerase II in the absence of drug were sequenced as well as 97 sites induced in the presence of doxorubicin. These sites were aligned relative to the base immediately 3′ to the cleavage sites (covalent linkage to topoisomerase II at the 5′-DNA terminus). Deviation of base distribution at a given position was determined by Chi-square analysis. A core region of 10 bases was found (between positions -3 and +7). The strongest bias was at position [-1]. In the presence of doxorubicin, the strongest base preference was A[-1]. Sites that did not have A[-1] had always T[+5]. Since positions [-1] and [+5] have diad symmetry relative to a topoisomerase II DNA double-strand break, requirement for A[-1] or T[+5] is equivalent to requirement for A[-1] at one of the cleavage sites of a DNA double-strand break. Hence, anthracycline-induced DNA cleavage was always at sites that had at least one A[-1]. This finding suggests that the binding of one anthracycline molecule at one of the cleavage sites of a DNA double-strand break is sufficient for topoisomerase II inhibition. Furthermore, it suggests that anthracyclines bind to the A[-1] of topoisomerase II-DNA complexes. In the absence of drug, the strongest base preferences were also at positions [-1] and [+5]. However, not-A and not-T were preferred at these positions. This finding is in agreement with the observation that anthracycline-induced DNA cleavage sites are never at strong enzyme cleavage sites in the absence of drug.

3. Structure-Activity Relationship of VP-16 derivatives with modifications of the essential phenolic ring:

The demethylepipodophyllotoxins, VP-16 and VM-26 are potent inhibitors of mammalian topoisomerase II. They differ in their glycosidic substituents which affect potency. VM-26 is approximately 10-fold more potent than VP-16 against purified topoisomerase II and is also 10-fold more cytotoxic in tissue culture. Both compounds have the same essential phenolic ring. We have now compared the activities of 4-methoxy, dihydroxy, and ortho-quinone

modifications of this ring. The 4-methoxy derivative (which has the same phenolic ring as podophyllotoxin, a tubulin inhibitor without effect upon topoisomerase II) did not inhibit purified topoisomerase II and was not cytotoxic. The two other compound were as active as VP-16 in topoisomerase II cleavage assays, indicating that some phenolic ring modifications can be made without loss of activity, but that complete methylation of the ring oxygens abolishes topoisomerase II inhibition.

4. DNA Sequence Requirements for Topoisomerase II-Induced DNA cleavage in the presence of Demethylepipodophyllotoxins (VP-16 and VM-26) and Acridines (*m*-AMSA):

The same methodology that was used to determine the basis for selectivity of drug action in the case of camptothecin and anthracyclines was applied to the demethylepipodophyllotoxins and acridines. First, we found that VP-16 and VM-26 induced the same cleavage sites and that VM-26 was 10-fold more potent than VP-16. 112 strong cleavage sites induced by VM-26 in SV40 DNA were analyzed in
order to determine the local base requirements for topoisomerase II inhibition. Strongest base preference was C[-1]. Sites that did not have C[-1] exhibited preference for G[+5]. Since positions [-1] and [+5] possess diad symmetry relative to a topoisomerase II DNA double-strand break, preference for C[-1] or G[+5] is equivalent to preference for C[-1] at one of the cleavage sites of a DNA double-strand break. Hence, VM-26- (and VP-16)-induced DNA cleavage was preferentially at sites that had at least one C[-1]. This finding suggests that the binding of one VM-26 (or VP-16) molecule at one of the cleavage sites of a DNA double-strand break is sufficient for topoisomerase II inhibition. Furthermore, it suggests that VM-26 and VP-16 may bind to the C[-1] of topoisomerase II-DNA complexes. In the case of *m*-AMSA, strongest base preference was A[+1]. Sites that did not have A[+1] exhibited preference for T[+4]. Since positions [+1] and [+4] have diad symmetry relative to a topoisomerase II DNA double-strand break, preference for A[+1] or T[+4] is equivalent to preference for A[+1] at one of the cleavage sites of a DNA double-strand break. Hence, *m*-AMSA-induced DNA cleavage was preferentially at sites that had at least one A[+1]. This finding suggests that the binding of one *m*-AMSA molecule at one of the cleavage sites of a DNA double-strand break is sufficient for topoisomerase II inhibition and that the drug interacts with the A[+1]. Taken together these finding suggest a common mode of topoisomerase inhibition by drugs. Drugs would stabilize topoisomerase-DNA complexes by binding inside the cleavage sites and forming a ternary complex. Classical DNA intercalation is not necessary for this binding to occur. Rather drugs would stack with the bases flanking the cleavage sites. Base selectivity of drug action would be dependent upon the drug polyring system.

5. Cloning of the topoisomerase I gene(s) from sensitive and drug-resistant Chinese hamster cells:

Eukaryotic topoisomerase I genes have only been cloned in yeast and human

cells but not in Chinese hamster cells. We have previously found two forms of topoisomerase I in Chinese hamster cells (100 kDa and 68 kDa), and made rabbit polyclonal antibodies against the 68 kDa form of the enzyme (Pommier et al, *J Biol Chem*, 1990;265:9418). In order to determine whether both enzyme were derived from a single gene we made a library from Chinese hamster DC3F cells and screened it using the 68 kDa antibodies. Sequencing of positive clones is in progress.

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 basis for DNA sequence selectivity of drug action. We are planning to investigate mitoxantrone and its analogs, and possibly a new family of drugs which have been rationally designed as topoisomerase II inhibitors.

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 and drug photoactivation could be used for these studies.

5. Clone the topoisomerase I gene(s) from camptothecin-resistant cells.

6. Map the locations of natural and drug-stimulated topoisomerase II sites in the integration and regulatory regions in the HIV LTR's.

Publications:

Sinha BK, Politi PM, Kerrigan D, Pommier Y. Structure-activity relations, cytotoxicity and topoisomerase II-dependent DNA cleavage induced by pendulum ring analogs of Etoposide, Eur J Cancer 1990;26:590-93.

Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier, Y. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerase I and II, Mol Pharmacol 1990;38:38-45.

Capranico G, Jaxel C, Roberge M, Kohn KW, Pommier Y. Nucleosome positioning as a critical determinant for the DNA cleavage sites of mammalian DNA topoisomerase II in reconstituted simian virus 40 chromatin, Nucleic Acids Res 1990;18:4553-9.

Capranico G, Kohn KW, Pommier Y. Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin, Nucleic Acids Res 1990;18:6611-19.

Jaxel C, Capranico G, Kerrigan D, Kohn KW, Pommier Y. Effect of local DNA sequence on topoisomerase I cleavage in the presence or absence of camptothecin, J Biol Chem, *in press*.

Pommier Y, Jaxel C, Kerrigan D, Kohn KW. Structure activity relationship of topoisomerase I inhibition by camptothecin derivatives: evidence for the existence of a ternary complex. 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

PROJECT NUMBER: Z01 CM 06170-07 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Histone H2A.Z Gene

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

PI: Christopher L. Hatch Sr. Staff Fellow LMPH NCI

Others: William M. Bonner Sr. Investigator LMPH NCI

COOPERATING UNITS *(if any)* Dr. Peter Vos, Laboratory of Viral and Molecular Pathogenesis, NINDS, NIH. Dr. Rufus Burlinghame, W. M. Keck Autoimmune Disease Center, Research Institute of Scripps Clinic, La Jolla, CA 92037.

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 organization and expression of the gene for H2A.Z. Transcription of the H2A.Z gene is modulated by the binding of protein factors to specific DNA sequence motifs located just upstream from the core promoter of the gene. These factors either enhance or repress the rate of transcription of this gene depending on the state of cell proliferation or differentiation. This gene, therefore, provides a useful model system to study the mechanisms by which the cell adjusts the rate of transcription of housekeeping genes to the appropriate level for each growth state. In addition, several observations made on the regulation of transcription of this gene suggest that this system may be utilized both to study how cellular proteins influence the level of transcription directed by the long terminal repeat of the integrated human immunodeficiency virus and how, in turn, the HIV transactivator protein, TAT, may affect the level of transcription of cellular genes.

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. We have also made observations which indicate that this system can be utilized to investigate the interplay between viral (HIV) and cellular proteins in gene regulation.

Methods:

1. Recombinant DNA techniques.

2. Assays of expression of transfected recombinant DNAs in mammalian cells.

3. Oligonucleotide synthesis and polymerase chain reaction.

4. DNase I footprinting, gel retardation, and uv cross-linking to analyze the binding of transcription factors to specific DNA sequences in the promoter region of the H2A.Z gene.

5. In situ hybridization of mRNA in sections of mouse embryos at different stages of development.

6. Immunological and biochemical techniques to purify the human H2A.Z protein after its expression in bacteria.

7. Use of two dimensional gel electrophoresis for identification of histone variant proteins.

Major Findings and Accomplishments:

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 cells and down-regulate the promoter in differentiated cells.

In order to study this upstream control region of the H2A.Z promoter, the techniques of DNase I footprinting and gel retardation have been utilized to determine the consensus DNA sequences to which the transcriptional control factors are binding and how these factors might differ in embryonic cells as opposed to other cell types. Three sequences, not reported elsewhere, have been found and are being characterized. A fourth sequence motif involved in the regulation of the rate of transcription of this gene appears to belong to a family of conserved cis-acting sequence elements identified recently in a variety of vertebrate genes, as well as in the human immunodeficiency virus long terminal repeat. In each of the cases studied to date, including this study, elements of this type reduce the rate of transcription directed by the promoters of both the homologous gene as well as those of heterologous genes. The molecular weights of the proteins binding to the cis-acting sequence elements of the human H2A.Z gene have been determined and we intend to proceed on to the cloning and characterization of these newly identified transcriptional control factors.

In order to examine whether or not the expression of the histone H2A.Z gene is up- or down-regulated at a particular developmental stage or in a specific tissue we have made a series of probes that allow by in situ hybridization specific analysis of the levels of H2A.Z mRNA present in the various tissues of mouse embryos (collaboration with Dr. Peter Vos, Laboratory of Viral and Molecular Pathogenesis, NINDS, NIH).

Transcription directed by the promoter of the human H2A.Z gene has been observed to be repressed by the TAT transactivator protein of the human immunodeficiency virus. In addition, one of the cis-acting sequence elements of the human H2A.Z gene upstream region is specifically bound by two cellular proteins and is very likely to be involved in the down-regulation of H2A.Z gene transcription. This element is highly homologous to a putative negative regulatory sequence element observed in the promoter regions of other genes, including those encoding lysozyme, vimentin, growth hormone, insulin, epsilon-globin, interleukin-2 receptor, as well as in the human immunodeficiency virus long terminal repeat (HIV LTR). Study of this family of negative regulatory elements and the factors that bind them will provide valuable information regarding the control of transcription of not only cellular genes but also of the latent integrated HIV genome in infected, asymptomatic patients.

Proposed Course:

1(a). To determine the roles in gene regulation of each of the novel transcription factors that bind to distinct sequence elements in the 5-prime flanking region of the H2A.Z gene.

1(b). To clone and characterize the above-mentioned transcription factors. This will provide us insight into the mechanism(s) by which these transcription factors influence the level of gene transcription and will allow us to search for other members of the family of genes that are regulated by these factors.

1(c). To study how the specific binding of nuclear protein(s) to conserved sequence elements in the promoter regions of the H2A.Z gene, other vertebrate genes (such as lysozyme, vimentin, epsilon-globin, interleukin-2 receptor), as well as the HIV LTR causes transcriptional repression.

1(d). To investigate how the HIV TAT protein directly or indirectly brings about a reduction in the level of transcription directed by the H2A.Z gene promoter.

2. To determine whether or not the expression of the H2A.Z gene exhibits any tissue or developmental stage specificity.

3. The cloned human H2A.Z cDNA will be expressed in bacteria and the H2A.Z protein will be purified and utilized for several purposes: firstly, to elicit antibodies in a special strain of mouse that are more likely to produce antibodies against such an evolutionarily conserved and poorly immunogenic protein (collaboration with Dr. Rufus Burlingame, Research Institute of Scripps Clinic), and secondly, to do x-ray crystallographic analysis of crystals of this histone in native complexes with the other histones and DNA (also with Dr. Burlingame). This latter approach will allow us to understand structurally how this histone variant might contribute to a different chromatin structure that is more easily transcribed.

Publications:

Hatch CL, Bonner WM. The human histone H2A.Z gene: sequence and regulation, J Biol Chem 1990;265:15211-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06172-07 LMPH

PERIOD COVERED: October 1, 1990 to September 30, 1991

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
	Patrick M. O'Connor	Visiting Associate	LMPH NCI
Others:	Michael Walton	Visiting Fellow	LMPH NCI
	Ann Orr	Microbiologist	LMPH NCI

COOPERATING UNITS *(if any)* Drug Synthesis and Chemistry Branch, DTP; Pediatric Oncology Branch, COP (Dr. Ian Magrath); Surgery Branch, COP (Dr. Richard Alexander); Biological Response Modifiers Program, FCRDC (Dr. D. Ferris)

LAB/BRANCH: Laboratory of Molecular Pharmacology, DTP, NCI

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.0	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

This project investigates the molecular selectivity of alkylating agents and other DNA-reactive drugs and explores the factors that govern selective cell killing by these drugs. (1) A systematic study was conducted of a series of acridine nitrogen mustards, prepared under DTP contract. These intercalating mustards are nearly as active in monofunctional as in bifunctional form and do not have to crosslink DNA to be active. The DNA sequence selectivity of these compounds was found to depend on the length of the connecting chain between the acridine and nitrogen mustard moieties. The optimum selective monofunctional derivative with a connecting chain length of 3 is a potential candidate for drug development. (2) The DNA sequence environment that favors interstrand crosslinking by various nitrogen mustards was studies using designed synthetic oligonucleotides. A major finding was that isophoramide mustard is more efficient than other standard nitrogen mustards in interstrand crosslinking. (3) The mechanism by which DNA crosslinking leads to cell death is being studied in Burkitt lymphoma cells that we had previously found to differ in sensitivity to HN2, but which were alike with respect to the production and repair of interstrand and DNA-protein crosslinks. Following treatment with HN2, the cells exhibited G2 delay and, at higher doses, retardation of DNA synthesis. The changes in the cyclin/cdc2 kinase system associated with G2 and mitosis are being investigated in order to localize the difference between the cell lines at the molecular level. So far, we have found that the difference resides prior to the suppression of cdc2 kinase activity.

PROJECT DESCRIPTION

Objectives:

1. Obtain basic information that could be used to identify or design improved alkylating agents.

    a. Determine the DNA sequence dependence and relative efficiencies of interstrand crosslinking by alkylating agents in synthetic oligonucleotides and in intact cells.

    b. Identify compounds that produce interstrand crosslinks selectively in certain DNA sequence regions, such as G-rich regions, and that produce a minimum of other chemical reactions in the cell.

    c. Identify drug candidates that combine monoalkylation and non-covalent DNA binding to yield optimum selectivity in certain genomic regions without producing crosslinks.

2. Determine the molecular bases for differential cytotoxic responses to a given DNA lesion challenge.

    a. Investigate the molecular events associated with DNA damage-induced cell cycle perturbation.

    b. Investigate the mechanisms leading to cell death following treatment with DNA damaging agents.

Methods:

1. High resolution gel electrophoresis and quantitation of band radioactivity using a Betascope (Betagen) analyzer.

2. Computer analysis of DNA alkylation site intensity patterns using custom designed programs.

3. DNA filter elution measurements of DNA damage and repair in cells.

4. Cytotoxicity assays by colony formation and cell proliferation rate measurements.

5. Cell cycle analysis by flow cytometry.

6. Analysis of enzymes and regulator molecules associated with cell cycle control (e.g. components of the cyclin/cdc2 kinase system), using analytic electrophoretic and immunochemical techniques.

Major Findings:

DNA intercalating mustards.

A homologous series of acridine mustards has been synthesized at the University of New Mexico under contract with the Drug Synthesis and Chemistry Branch, DTP. The compounds consist of an acridine moiety (which binds to DNA by intercalation between base pairs) linked by a hydrocarbon chain of variable length to a mono or bifunctional nitrogen mustard. The synthetic preparations have been completed for the intended series which includes connecting chain length of 2 through 6 linked to N,N-chloroethylmethylamine, N,N-chloroethylethylamine or N,N-bis(2-chloroethyl)amine. The compounds are being tested in the cell screen in order to determine the dependence of cytotoxic potency and cell line selectivity on connecting chain length and on mono versus bifunctionality of alkylation. Preliminary results using the COMPARE program suggest that the compounds have a distinctive differential cytotoxicity pattern which is distinguishable from the patterns produced by standard alkylating agents or by other drugs that have been tested (Dr. Kenneth Paul, personal communication).

The DNA sequence selectivity studies of the compounds in the promoter region of the c-myc gene have been completed and are being prepared for publication. The major finding is that optimum sequence selectivity for successive G's is obtained with a connecting chain length of 3. With increasing chain length, the sequence selectivity becomes successively reduced.

DNA interstrand crosslinking by nitrogen mustards in synthetic oligonucleotide duplexes.

Synthetic oligonucleotides were designed to determine the effects of base sequence context on the efficiency of interstrand crosslinking by various nitrogen mustards. The oligonucleotides had only a single G in each strand, so that the locations of the crosslinks would be unambiguous. Sequencing assays verified alkylation of the G's. The unanticipated preference of HN2 for crosslinking in 5'GNC3' sequences was confirmed. Crosslinking in the previously assumed sequence 5'GC3' was very weak. In addition to crosslinks, multiple single-strand alkylations were observed, raising the possibility that there may be significant alkylation of A's under our reaction conditions. There is probably little or no crosslinking via A's, however, because very little crosslinking occurred in 5'GC3' sequences. Nevertheless, this question will be further investigated using a greater variety A/T sequence patterns.

Crosslinking of 5'GNC3' sequences was HN2 concentration dependent, reaching a maximum of 8.5% of the total alkylated molecules. The A/T base sequence surrounding the 5'GNC3' site had a small but reproducible influence on the efficiency of crosslink production. Now that the crosslinking site 5'GNC3' has been verified, these investigations will be extended to oligonucleotides

having multiple G's in each strand, so as to test our hypothesis that crosslinking may be most efficient in G-rich regions.

Several nitrogen mustards were compared for their ability to crosslink the initial set of oligonucleotides (5'GNC3' or 5'GC3' surrounded by A's and T's). Melphalan showed no detectable crosslinking of these sequences. Studies in intact cells previously showed that melphalan produces less DNA interstrand crosslinking than does HN2, but the failure to detect any crosslinks in the current set of oligonucleotides may be due to a relatively low efficiency of crosslinking in these particular sequences. We will test melphalan again in oligonucleotides containing runs of G's in each strand.

Isophosphoramide mustard gave a particularly striking result, in that the yield of crosslinks in 5'GNC3' was as high as 12% with relatively few monoalkylation products. This yield was much higher than that produced by phosphoramide mustard (the reactive product of cyclophosphamide). There was no detectable crosslinking in 5'GC3'. These findings are attributable to the longer crosslink connecting chain between alkylating groups in isophosphoramide mustard compared with standard bis(2-chloroethylamine) type nitrogen mustards. Alkaline elution studies are in progress to determine whether this high efficiency for interstrand crosslinking by isophosphoramide mustard is operative in intact cells.

Chemosensitivity determinants in Burkitt lymphoma cell lines.

Burkitt lymphoma cell lines, obtained from the laboratory of Dr. Ian Magrath (Pediatric Oncology Branch, COP), were tested for differences in sensitivity to nitrogen mustard (HN2). The sensitivities of 11 cell lines was found to vary over a 5-fold range with respect to the drug concentration required for a given degree of growth inhibition. The lines all had similar growth rates and all had translocations of the c-myc gene on chromosome 8 to the immunoglobulin H-chain loci on chromosome 14. Drug sensitivity did not correlate with the presence of absence of the Epstein-Barr viral genome, with the geographic origin of the tumor, or with the precise positions of the 8;14 translocation breakpoints. The 2 most sensitive lines were further studied in comparison with the 2 least sensitive lines. DNA alkaline elution measurements of these cell lines following a given HN2 dosage showed that all 4 lines were similar in the extent of DNA interstrand crosslink or DNA-protein crosslink production, as well as in the rate of repair of these lesions. The observed differences between the cell lines in DNA lesion production and repair were within experimental error and could not account for the 5-fold sensitivity differences. These cell lines therefore are suitable for investigation of factors that may influence the cytotoxic consequences of a given chemical insult to the genome.

The effects of HN2 on progress through the cell cycle were determined by flow cytometry (with help from Dr. R. Alexander, Surgery Branch, COP). At equimolar doses, the sensitive cells show retardation in S-phase, while the less sensitive cells accumulate in G2. Preliminary data at equitoxic doses showed only minor differences between the cell lines. It seems therefore that the events that determine cytotoxic sensitivity are taking place at some point prior to the actual cell cycle events which reflect the degree of cytotoxicity expression.

Mechanism of DNA-damage-induced G2 delay.

Delay in the G2 phase is frequently reported following treatment with a variety of DNA damaging drugs. There is evidence that the degree of G2 delay can influence cytotoxicity. In the studies of Burkitt lymphoma cells described above, G2 delay is prominent, although perhaps not determinative of differential killing in these particular cell lines. Investigation of the molecular events associated with G2 phase however may be revealing in either case. If G2 delay is not itself determinative of differential toxicity of these cell lines, it must be distal to the critical events, in which case it may be possible to work backwards towards the critical events from events that are known to control G2.

We have begun by examining the cyclin/cdc2 kinase system in these cells, with generous assistance from Drs. Douglas Ferris and Dan Longo of the BRMP in Frederick who provided reagents and help in setting up assays. Treatment with HN2 was found to be followed by inhibition of immunoprecipitated p34(cdc2) kinase activity, as measured by phosphorylation of histone H1. This is consistent with the known cell cycle relationships of p34(cdc2) kinase activity. Also consistent with these relationships was the observation of a burst of H1-kinase activity when the G2-arrested cells eventually progressed through mitosis. The altered p34(cdc2) kinase activity after HN2 treatment was not due to changes in the amount of p34(cdc2) protein, which remained unchanged by Western blot analysis. Since p34(cdc2) kinase activity has been reported to be negatively regulated by tyrosine phosphorylation, we investigated the effect of DNA damage on the level of p34(cdc2) phosphotyrosine. Polyacrylamide gel electrophoresis and Western blotting with p34(cdc2) antisera resolved 3 bands: tyrosine phosphorylated, serine/threonine phosphorylated and unphosphorylated forms of the enzyme. The tyrosine phosphorylated form was found to increase as HN2 treated cells became arrested in G2, while the serine/threonine form seemed to remain relatively constant. These early experiments already suggest that the cytotoxic determinant event precedes enhanced p34(cdc2) tyrosine phosphorylation in the G2 delay process.

Proposed Course:

1. Continue the studies of interstrand crosslinking by nitrogen mustards in oligonucleotides to determine the effects of G-rich surroundings in the sequence. Test the efficiency of interstrand crosslinking by isophosphoramide mustard in intact cells by alkaline elution and determine the production and repair of interstrand crosslinks in individual genes.

2. Compare alkylating agents that react selectively in G-rich regions of the genome with new DNA crosslinkers that react selectively in A-rich regions, with respect to biochemical events associated with cell cycle perturbation and cell death.

3. Further develop the analysis of the cyclin/p34(cdc2) kinase system in Burkitt lymphoma cells lines exhibiting different cytotoxic responses to a given DNA lesion challenge. Use monoclonal antibodies to the phosphotyrosine form of p34(cdc2) to confirm and further study the specific changes that follow drug treatment. Measure cyclin B in cells following drug treatment.

4. Initiate studies of the mechanisms of cell death in selected cell lines treated with DNA crosslinkers.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06186-05 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

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

PI:	Vilhelm A. Bohr	Medical Officer, Sr. Investigator	LMPH NCI
Others:	Alfred May	Microbiologist	LMPH NCI
	Cynthia Myers	Biologist	LMPH NCI
	Michele Evans	Medical Staff Fellow	LMPH NCI
	Florence Larminat	Visiting Fellow	LMPH NCI
	Tinna Stevnsner	Guest Researcher	LMPH NCI
	Weiping Zhen	Visiting Fellow	LMPH NCI

COOPERATING UNITS *(if any)*
Dr. Rodney Narin, M.D. Anderson; Dr. Jay Robbins, NIH; Dr. Michael Gottesman, NIH; Dr. Michael Potter, NIH

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Laboratory of Molecular Pharmacology, DTP, DCT

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	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.)*

Our objective is to study the DNA damage and repair in various individual genes and in non-coding sequences with the genome. This is correlated to more traditional studies of DNA repair as an average over the genome. It is now established that there is intragenomic heterogeneity of DNA repair. Findings have indicated that active genes are preferentially repaired in mammalian cells and that determinations of DNA repair in specific genes are important for correlations to biological endpoints and to risk assessments. Whereas our earlier studies were limited to UV as a damaging agent, we have now developed methods to study DNA damage and repair after a certain carcinogens and cancer chemotherapeutics that react with the DNA. We are studying the DNA repair of genes in a number of human, cancer prone DNA repair deficient syndromes and in various human and rodent mutant cell lines, some of which are transfected with repair genes. We are also investigating the role of DNA repair in multidrug resistance.

Professional Personnel (Continued):

Charles Link	Medical Staff Fellow	LMPH NCI
Miroslav Pirsel	Guest Researcher	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, cancer chemotherapeutics, ionizing irradiation and oxidative damage.

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 resistant cell lines.

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. Electroporation. 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 for correlations to other biological end points such as resistance to UV damage. Changes in preferential DNA repair could have profound effects on such parameters without noticeably altering overall genome repair levels since the vital regions only constitute a very small fraction of the genome.

We have found considerable differences in the repair efficiency of different genes within the same cell. The constitutively transcribed protooncogene c-abl is much more efficiently repaired than the transcriptionally silent proto-oncogene c-mos. In experiments measuring the repair in genes which can be modulated with regard to activity, results have further suggested that when metallothionein genes which are normally inactive become activated, considerable corresponding increases in DNA repair efficiencies can be detected. These findings suggest a positive correlation between DNA repair efficiency and transcriptional activity in a gene. Lately, however, we have obtained results that would suggest that preferential DNA repair of a certain gene can be independent of the level of transcription.

Major Findings:

DNA damage and repair in genes after treatment with carcinogens, ionizing irradiation and chemotherapeutics.

With the use of the ABC excinuclease, we have developed methodology to quantitate cisplatinum intrastrand adducts, 4NQO, IQ (a highly mutagenic, heterocyclic compound) and NAAAF. We have been able to demonstrate preferential DNA repair of cisplatinum adducts in the CHO DHFR; NAAAF adducts were not preferentially repaired. In addition an assay has been developed to directly detect the sites of alkylation damage in DNA. In this assay, the DNA is nicked at alkylated sites by depurination and alkaline hydrolysis, and the frequency of alkylated sites in genes can then be measured. We have recently found that adducts formed after treatment with nitrogen mustard are initially formed at different frequencies in different genomic regions and that they are preferentially repaired in the CHO DHFR gene. Cisplatin intrastrand adducts can be quantitated within genes using the ABC excinuclease, and we have been able to detect cisplatin interstrand crosslinks using a denaturation-reannealing gel assay. Lately, we have been able to assay for X-ray induced single strand breaks in our hamster DHFR assay.

Studies of preferential DNA repair and strand specificity of DNA repair in the DHFR gene after treatment of CHO cells with UV and chemotherapeutics.

We have studied DNA damage and repair in the DHFR gene and in a noncoding region in CHO cells after different kinds of damage. CHO cells were UV irradiated or treated with nitrogen mustard, dimethyl sulphate or cisplatin, the DNA was extracted and after specific reactions, it was Southern blotted and probed. The frequency of pyrimidine dimers, 6-4 photoproducts, N7 alkylations, cisplatin intrastrand adducts and cisplatin interstrand crosslinks was determined in the different genomic regions. Pyrimidine dimers, 6-4 photoproducts, cisplatin intrastrand lesions and nitrogen mustard are preferentially repaired, i.e. there is significantly more efficient repair in the coding than in the noncoding region. In contrast, dimethylsulphate and cisplatin interstrand crosslinks are not preferentially repaired. The membranes were then reprobed with strand specific riboprobes for the DHFR gene to detect whether damage and repair was preferential in the coding strand. There are marked differences in the repair characteristics at the gene level for these different lesions. The most distinct strand specificity (and the most distinct preferential repair) is seen for pyrimidine dimers. For the other lesions there is little or no strand specificity. There may be a correlation between the extent of transcription blocking by a lesion and the strand specificity of its repair. Preferential DNA repair may be dependent upon degree of chromatin structural change that a DNA damaging agent inflicts.

Repair of genes in human disease.

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 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 specific 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. 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 these repair deficient human cells, and that the limited activity may be directed towards vital regions.

Because of our interest in the correlation between repair and transcription and the possible role of DNA repair in drug resistance, we have studied gene specific repair in the human cervical cancer KB cells. The KB 3-1 parental line has very low levels of MDR1 expression, but its multidrug resistant derivative, KB 8-5, demonstrates increased levels of MDR1 expression without MDR1 gene amplification. We have measured gene specific DNA repair of ultraviolet light induced cyclobutane pyrimidine dimers in both KB cell lines. Repair was studied in a 23 kb fragment of the housekeeping gene, DHFR, in a 23 kb fragment of the MDR1 gene, in the inactive deltaglobin gene and in a 10 kb fragment of the topoisomerase II gene. Preliminary data suggest differences in the observed levels of repair in the MDR1 gene between the KB3-1 and KB8-5 cell lines that might be related to the levels of gene expression.

Effect on gene repair of inhibitors of topoisomerases and polymerases.

In order to investigate which enzymes are involved in specific gene repair we studied the effect of specific inhibitors on the preferential DNA repair of UV induced pyrimidine dimers in the hamster DHFR gene. None of the following agents had any effect on the gene specific repair: Aphidicolin, 3-AB, hydroxyurea, VP-16, merbarone, camptothecin and beta-lapachone. However, all of these agents inhibited the genomic replication and most of them affected the overall genome repair as measured by repair replication. One compound, caffeine, had some inhibitory effect on the DNA repair in the DHFR gene. This inhibition is seen at doses of 0.5 and 10 mM caffeine present in the media

during 8 and 24 hours of repair. We are currently evaluating the repair in the non-transcribed 3′ flank of the gene the strand specificity of the repair to further describe the mechanism(s) involved.

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) are added together, then 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.

Formation and repair of cisplatin lesions in specific genes in cisplatin sensitive and resistant human ovarian cancer cell lines.

We have examined the formation and removal of cisplatin (CP) intrastrand adducts and interstrand crosslinks in specific genes (DHFR, MDR1, deltaglobin) in two CP-resistant human ovarian cancer cell lines. The intrastrand adducts were detected with the use of ABC excinuclease followed by a previously described method for Southern blot quantitation. The crosslinks were detected by a denaturation-renaturation procedure followed by gel-electrophoresis and Southern analysis. We found that cisplatin crosslinks were repaired much faster from specific genes in CP-resistant cell lines as compared to their parental CP-sensitive cell lines, while removal of cisplatin intrastrand adducts was similar in both the CP-resistant and the CP-sensitive cell lines. Since the rate of interstrand crosslink removal from the overall genome, as measured by alkaline elution, was similar in all cell lines, our findings suggest that DNA repair in specific genes may be important in cellular resistance.

DNA repair in the c-myc protooncogene in mouse B lymphocytes.

We have examined DNA repair kinetics in selected regions of the c-myc and DHFR genes in normal B lymphoblasts derived from inbred mice that are resistant (DBA) or susceptible (BALB/c) to induced plasma cell tumors. DNA repair kinetics are similar in the protein-encoding portion of c-myc and in the DHFR gene in these cells. However, in the 5′ flank of c-myc (a hotspot for translocation breakpoints in BALB/c plasmacytomas) BALB/c cells are repair deficient when compared to DBA cells. What is more, in the protein-encoding portion of c-myc, damage is selectively removed from only the transcribed strand. No repair is detected in the non-transcribed strand. In contrast, DNA repair in the 5′ flank of c-myc is not strand specific; in DBA cells, damage is rapidly removed from both DNA strands, while in BALB/c cells no repair was detected in either strand in the 5′ flank. These findings provide new insights into the details of gene-specific and strand-specific DNA repair and suggest that DNA repair and B cell neoplastic development may be closely associated.

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 molecular biology of preferential DNA repair, and 2) DNA repair in human disease, relation to cancer risk and therapy.

AIDS-Lymphoma related projects.

We have started experiments with the objective of elucidating the role of DNA repair in AIDS or lymphoma. This involves a number of different studies: We will investigate whether the DNA repair at the level of the overall genome or in the c-myc gene region is altered in infected cells. We will examine the repair in the 5′-flanking region of c-myc in normal human and in Burkitts cells to search for repair differences that are similar to those described above for the murine c-myc repair. Also, we will measure the DNA repair in the HIV genome itself.

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.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06192-03 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

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)*

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Others:	Cecilia Mannironi	Visiting Fellow	LMPH NCI	(departed 3/1/91)
	Christopher Hatch	Sr. Staff Fellow	LMPH NCI	
	Duane Pilch	Staff Fellow	LMPH NCI	
	Concepcion Muneses	Chemist	LMPH NCI	

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.1	0.9

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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 primary objective of this project this year has been to determine how a replication-independent histone protein species, H2A.X, results from an apparently replication-dependent gene. The H2A.X gene is the only known situation in which this occurs. The study is of potential importance because understanding the mechanism of this uncoupling is a step towards our goal of being able to manipulate the balance between histone and DNA synthesis for therapeutic benefit.

The mRNA is 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. In addition to the altered processing of the H2A.X compared to the H2A.1 transcript, there is also altered regulation of the mRNA stability. The major effort at this time is delineating those sequences and factors which lead to the altered behavior of the H2A.X transcript by analyzing the transcripts of chimeric H2A.X/H2A.1 or otherwise changed H2A.X genes transfected into various cell types. This type of analysis will allow us to progressively localize relevant sequences. One sequence has been found and is being studied.

The altered behavior of the H2A.X transcript enables it to escape tight replication-dependence. Elucidating this mechanism may allow the development of techniques disrupting the tight control of histone and DNA synthesis, leading to selective death of tumor cells.

Project Description

Introduction:

Mammalian cells contain four histone H2A isoprotein species, called H2A.1, H2A.2, H2A.Z and H2A.X. In proliferating cells, the former two are synthesized in concert with chromatin replication, while the latter two are synthesized throughout the cell cycle. In quiescent cells, all four isoprotein species are synthesized at much lower rates but in relative amounts similar to those in proliferating cells. H2A.X represents about 10-15% of total H2A in most mammalian cell lines and tissues. 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 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 and (2) to study the factors regulating the altered stability of the H2A.X mRNA.

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 human H2A.X Gene

The H2A.X gene clone bs2axsx contains 500 bp upstream through the promoter region and about 1000 bp downstream from the polyA attachment site. The H2A.X gene does not seem to contain any introns. As part of this work, the human H2A.1 gene promoter was sequenced. The H2A.X gene contains TATAA and CCAAT boxes in similar positions to those in the H2A.1 gene. In addition there is another conserved sequence which we have not been able to assign by sequence homology to any known factors.

2. Sequences involved in the altered processing and stability of H2A.X transcripts

Both the human H2A.X and H2A.1 genes efficiently transcribe when transfected into CHO cells. We are using this system to prepare different gene constructs to find sequences important in the altered regulation of H2A.X gene products. One notable feature of the H2A.X gene is a 10 bp sequence at the translation

termination codon which is complementary to one half of the stem-loop at the 3′ end of the mRNA. When a similar feature was inserted into an H2A.1 gene which was then transfected into CHO cells, the processing of the mRNA was dean, suggesting that such features do affect transcript processing in vivio.

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. A manuscript describing these findings has been submitted.

Significance to Biomedical Research and to the Program of the Institute

Thus the H2A.X gene is of potential importance because it is the only known situation in which the synthesis of a histone protein from an apparently replication-dependent histone gene is uncoupled from replication in somatic cells. Understanding the mechanism of this uncoupling is a step towards our goal of being able to manipulate the balance between histone and DNA synthesis for therapeutic benefit.

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. Prepare artificial genes to study the factors involved in the regulation of the H2A.X mRNA processing.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07102-16 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Tubulin as a Site for Pharmacologic Attack

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

PI:	E. Hamel	Senior Investigator	LMPH, NCI
Others:	R. Bai	Visiting Associate	LMPH, NCI
	Z. Getahun	Visiting Fellow	LMPH, NCI (Departure 1/91)
	S. Grover	Visiting Fellow	LMPH, NCI
	C. M. Lin	Biologist	LMPH, NCI

COOPERATING UNITS *(if any)* 1) G. Pettit, Arizona St. Univ; 2) L. Jurd, USDA; 3) L. Powers, Ricerca Corp.; 4) A. Brossi, NIDDK; 5) M. Banwell, Univ. of Melbourne; 6) N. Nguyen, FDA; 7) J. Jiang, Dupont Co.; 8) S. Friedman, LBC, NCI; 9) M. Cushman, Purdue; 10) R. F. Luduena, Univ. Texas, San Antonio;

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	3.3	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 many classes of compounds. Analogs of combretastatin A-4 were examined to determine important structure-activity relationships, and studies to determine the optimal bridge length between phenyl rings were completed. 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, compounds which do not appear to interact strongly 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-styrylquinazolin-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 DDP derivatives appear to bind at a unique binding site on tubulin. A radiolabeled A-ring modified analog of colchicine was synthesized. It reacts covalently with beta-tubulin. We initiated studies with photoactive and chemically reactive analogs of taxol which should ultimately prove useful in defining the taxol binding site on tubulin. The DTP drug screening data base was examined using the COMPARE program with known antimitotic compounds as seeds to yield multiple leads for new antimitotic agents. The most potent were halichondrin B and homohalichondrin B. These agents were shown to be noncompetitive inhibitors of vinblastine binding to tubulin.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Cooperating Units (Continued):

11) D. Kingston, Virginia Polytechnic Inst.; 12) K. Paull, ITB, NCI

Microtubules, cellular organelles critical for cell division, are sensitive to a number of antineoplastic drugs. These drugs all cause cells to accumulate in prometaphase, disrupting mitosis, for microtubules are the major component of 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 previously studied in detail. Studies to define the optimum length of the hydrocarbon bridge between the two phenyl rings of the combretastatins were completed (a 2-carbon bridge is optimal). Dr. M. Cushman and his collaborators at Purdue University have synthesized a large series of analogs of combretastatin A-4. Many have significant cytotoxic activity, and we are evaluating their antimitotic and antitubulin activity. Together with our earlier work with Dr. Pettit, this should enable us to obtain a thorough structure-activity understanding of this important natural product.

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 radiolabled 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 beta-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. Recently a dolastation 10-resistant Chinese hamster ovary (CHO) cell mutant has been isolated which appears to have an altered tubulin species. Its characterization has been undertaken.

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. Supplies of dolastatin 14 are too limited at present for detailed studies. Dolastatin 15 was found to have weak effects on in vitro tubulin polymerization (about 1/20-th the potency of dolastatin 10). Immunofluorescence studies with CHO cells, however, demonstrated that the microtubule system disappeared following dolastatin 15 treatment. This is the typical finding with antitubulin compounds. We conclude that the antimitotic properties of dolastatin 15 derive from its interaction, however weak, with tubulin. The agent may be taken up with unusual affinity by cells in culture, or it may be metabolized to a more active form.

Dolastatin 11 appears to act by an entirely novel, and as yet not understood, mechanism. Instead of cells arrested at mitosis, dolastatin 11 causes the appearance of large numbers of multinucleated L1210 cells. These cells have a hyperploid (> 4N) DNA content. Normally bipolar glial cells treated with dolastatin 11 become multipolar with multiple processes containing what appear to be thick bundles of microtubules. CHO cells produce short processes and become multinucleated. 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. We have been able to derivatize one of Dr. Brossi's active analogs to create a highly active inhibitor of tubulin polymerization which is chemically reactive with the protein, thus creating an affinity label for the colchicine site of tubulin. We have prepared 3-demethyl-3-chloroacetyl-thiocolchicine in both nonradiolabeled and radiolabeled forms. This compound reacts preferentially with beta-tubulin (alpha:beta ratio of radiolabel about 1:5), thus indicating that the A ring of colchicine is probably in close contact with beta-tubulin. We are currently attempting to isolate peptides to identify the binding site in greater detail.

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 for screening, and some of these were found to have antineoplastic activity. We have found that a number of these drugs inhibit mitosis in mammalian cells in culture and the polymerization of tubulin in vitro. 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 we have succeeded in demonstrating interaction of these compounds by equilibrium chromatography (by the Hummel-Dreyer technique). The dissociation constant for the more active DPP derivative is about 20 uM, higher than expected considering its relative activity as an inhibitor of microtubule assembly. Binding of the compound was substantially enhanced by higher reaction temperature, and the binding of the DPP derivative was inhibited by colchicine, podophyllotoxin, vinblastine, and maytansine, but not by combretastatin A-4. Earlier results had found no effect of DPP derivatives on the binding of radiolabeled vinblastine, maytansine, or colchicine, suggesting that the DPP derivatives bound at a different drug binding site on tubulin. We are presently trying to resolve the apparent contradiction obtained when different radiolabeled ligands are used.

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 previously performed a structure-activity analysis of the approximately forty available analogs. We have now used the more active analogs to study the mechanism of interaction of the SQZ derivatives with tubulin. These agents bind at the colchicine site as competitive inhibitors of the binding of radiolabeled colchicine to tubulin. Both the binding and dissociation

reactions are extremely rapid, and the rate constants for these reactions for the SQZ derivatives appear to be greater than any yet described for colchicine site drugs. In fact, the SQZ derivative interactions with tubulin are so rapid that inhibition of colchicine binding can only be convincingly demonstrated at short reaction times and a relatively low colchicine to tubulin ratio.

7) Dr. D. G. I. Kingston of Virginia Polytechnic Institute is presently synthesizing chemically reactive and photoactive analogs of taxol. Two nonradiolabeled analogs have been prepared, and we demonstrated that these analogs retain the biological activity of taxol. Our specific goal is to use radiolabeled preparations to define the binding site of taxol on tubulin. Covalently modified tubulin peptides will be isolated in our laboratory and sequenced by Dr. Kingston.

8) We have begun a collaboration with Dr. K. D. Paull of the Information Technology Branch, DTP, who has developed a computer program (called "COMPARE") which permits analysis of the DTP drug screening data for patterns of differential cytotoxicity. In brief, the screening data base is compared to a drug of known mechanism of action to select new agents with similar patterns of toxicity toward the sixty cell lines used in the screen. The COMPARE program is quite effective in providing leads for new antitubulin agents when probed with known antimitotic agents such as colchicine, vinblastine, podophyllotoxin, combretastatin A-4, or taxol. It led us to two potent sponge-derived complex natural products, halichondrin B and homohalichondrin B (isolated by Dr. G. R. Pettit). These proved to be inhibitors of tubulin polymerization and mitosis, and they are noncompetitive inhibitors of the binding of vinblastine to tubulin. COMPARE also indicated approximately twenty other agents should be evaluated as potential antitubulin agents. These studies are in progress, and initial results are very promising.

Publications:

Bai R, Pettit GR, Hamel E. Structure-activity studies with chiral isomers and with segments of the antimitotic marine peptide dolastatin 10, Biochem Pharmacol 1990;40:1859-64.

Bai R, Pettit GR, Hamel E. Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites, J Biol Chem 1990;265:17141-49.

Kang GJ, Getahun Z, Muzaffar A, Brossi A, Hamel E. N-acetylcolchinol O-methyl ether and thiocolchicine, potent analogs of colchicine modified in the C ring: evaluation of the mechanistic basis for their enhanced biological properties, J Biol Chem 1990;265:10255-9.

Muzaffar A, Brossi A, Hamel E. Partial synthesis and antitubulin activity of minor Colchicum alkaloids: N-deacetylcolchicine and 1-demethylspeciosine (speciocolchine), J Nat Prod 1990;53:1021-4.

Muzaffar A, Hamel E, Brossi A. Reaction of colchiceinamide with phosgene and with thiophosgene: structures and antitubulin activity of tetracyclic oxazolones, oxazolethiones and thiazolones of the colchicine series, Heterocycles 1990;31:2037-40.

Cushman M, Nagarathnam D, Gopal D, Chakraborti AK, Lin CM, Hamel E. Synthesis and evaluation of stilbene and dihydrostilbene derivatives as potential anticancer agents that inhibit tubulin polymerization, J Med Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07179-06 LMPH

PERIOD COVERED
October 1, 1990 to September 30, 1991

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:	Z. Getahun	Visiting Fellow	LMPH, NCI	(departed 1/91)
	S. Grover	Visiting Fellow	LMPH, NCI	
	C. M. Lin	Biologist	LMPH, NCI	

COOPERATING UNITS *(if any)*
Dr. P. Skehan, LDDRD, 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:
1.0	1.0	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 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 (MAPs). The effects of nucleotides on the stability of microtubules continued to be examined, as were conditions to optimize the separation of alpha-tubulin and beta-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. Roles of divalent cations in nucleotide binding to tubulin, in tubulin polymerization and in polymer stability 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. Additional cations were also evaluated for effects on these reactions. A project to define the disulfide bridges in tubulin and in two major classes of MAPs was completed, with the observation that all cysteine sulfhydryl groups in these proteins were free. Studies were initiated to determine whether free sulfhydryls played a role in the stability of either tubulin or MAPs. We have succeeded in synthesizing 2′,3′-dideoxyguanosine 5′-[alpha, beta-methylene]-triphosphate, and we are evaluating its effects on microtubule assembly.

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.

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 LDDRD, 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. 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 Be^{2+} 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 reactions, 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. With Sn^{2+}, on the other hand, significant amounts of GTP were not hydrolyzed, but the polymer was cold labile and tubulin did not precipitate.

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 Be^{2+}.

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 unambiguously the essential triphosphate. Recent important findings are that high concentrations of Mg^{2+} drastically destabilize microtubules at low GTP concentrations, and that GTP breakdown affects microtubule stability differently with MAP-2 as opposed to tau protein.

We plan to exploit the known ability of guanosine 5′-[alpha,beta-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-beta-methylene modifications. We have succeeding in synthesizing 2′,3′-dideoxyguanosine 5′-[alpha, beta-methylene]triphosphate, and its effects on microtubule assembly are being evaluated.

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 beta-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-beta-tubulin dimer, we find that there are no disulfide bonds in tubulin, despite a cysteine content of twenty residues in the dimer (eight in beta,

twelve in alpha). In addition we have found no evidence for disulfide bonds in MAPs fractions enriched for tau protein or MAP-2. We are attempting to determine the role of sulfhydryl oxidation-reduction state on the stability of tubulin and MAPs.

We are continuing to devote a great deal of attention to MAPs. We are particularly interested in a MAP which causes the formation of microtubule bundles (distinct microtubules which aggregate laterally). The active component (termed MAP-TB) appears to be present in MAP preparations in extremely small amounts. Although it is highly stable, it has proven more difficult to purify than anticipated. Despite DEAE-cellulose chromatography, ammonium sulfate fractionation, heat-treatment, hydroxyapatite chromatography, and HPLC chromatography (ion-exchange and gel filtration), the purest preparations remain disappointingly heterogeneous on polyacrylamide gel electrophoresis. Tubulin affinity chromatography may be useful in its purification.

Publications:

Hamel E, Lin CM, Kenney S, Skehan P. Highly variable effects of beryllium and beryllium fluoride on tubulin polymerization under different reaction conditions: comparison of assembly reactions dependent on microtubule-associated proteins, glycerol, dimethyl sulfoxide, and glutamate, Arch Biochem Biophys 1991;286:57-69.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07184-02 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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.	Sr. Investigator	LMPH	NCI
Others:	I. Alamo	Microbiologist	LMPH	NCI
	M. C. Hollander	Microbiologist	LMPH	NCI
	J. Jackman	ERDA Fellow	LMPH	NCI

COOPERATING UNITS *(if any)*

NIA, Baltimore, MD (N. Holbrook); Univ. of Chicago (R. Weichselbaum); 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)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.15	3.65	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

In bacteria and yeast, many of the genes important in the cellular responses to DNA damage are induced by such damage. The focus of this project is the study of similar processes in mammalian cells and the role of DNA-damage responses in determining the cellular sensitivity to cytotoxic agents, such as used in cancer therapy. Using specialized cloning procedures which we developed, cDNA clones for more than 20 novel DNA-damage-inducible (DDI) genes have been isolated and sequenced. In both bacteria and eukaryotes, one effect of DNA damage is the transient inhibition of DNA synthesis and cell growth; such delays can have a protective effect since mutants lacking growth arrest responses are hypersensitive to certain DNA-damaging agents. We have found that 5 of our clones encode 5 different hamster genes that are coordinately induced either by DNA-damaging agents or by other cell treatments that induce growth arrest such as serum reduction; these genes have been designated *gadd* (growth arrest- and DNA damage-inducible). In collaboration with D. Nebert and N. Holbrook, we now have good evidence that the *gadd* genes are coordinately regulated. Two of the *gadd* 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. Further efforts will focus on the regulation of the DDI genes and their functions.

Cooperating Units (Continued):

Lawrence Livermore Natl. Lab., Livermore, CA (L. Thompson); M.D. Anderson, Univ. of Texas, Smithville, TX (R. Nairn); N. C. I. (J. Robbins); Georgetown Univ. (M. Smulson); Emory Unov. (P. Doetsch).

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

Genomic clones for two of the *gadd* genes, *gadd45* and *gadd153*, have been isolated from both human and hamster cells, and the genes with their 5' regulatory regions have been sequenced. The sequences of the coding regions for the human and rodent homologues of these genes are highly conserved. The genes have probably been highly conserved throughout vertebrate evolution since their homologues can be detected in fish DNA using high stringency hybridization (in collaboration with R. Nairn). Such a high degree of evolutionary conservation usually indicates that the products of such genes have important cellular functions. The regulation of these genes has also been well-conserved since both the *gadd45* and *gadd153* genes have been found to be DNA-damage inducible and growth-arrest inducible in every mammalian cell tested. In addition, the promoters of the *gadd45* and *gadd153* genes contain limited 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. By deletion mapping, regions of the these promoters containing DNA-damage-responsive elements have been identified. Interestingly, one such region in the *gadd45* promoter shows the greatest homology with the *gadd153* promoter.

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 collaboration with R. Weichselbaum, we recently have found that this x-ray-responsiveness is lost in many human tumor cell lines. For example, the response was found in all 12 non-tumor cell lines or strains examined, while it was retained in only 6 of 17 tumor lines. This finding, that a gene associated with growth arrest loses its response to x rays in most tumor cells, may provide a clue to alterations in growth control mechanisms in tumor cells. Since negative growth control responses may have a protective role for cells exposed to cytotoxic agents, our findings may have therapeutic relevance.

The regulation of the *gadd* genes by growth arrest has been investigated and compared to other genes involved in negative growth control. Genes, such as the *gas* (growth-arrest specific) genes, prohibitin, p53, Rb (retinoblastoma), and other tumor suppressors, show increased expression in growth-arrested cells or have been shown to play a role in negative growth control. In contrast to the *gadd* genes, none of these other genes were found to be DNA-damage inducible. In the case of the *gas1* gene, it was strongly induced by either reduced serum, medium depletion (medium starvation), or contact inhibition in 3T3 cells but not by methylmethane sulfonate (MMS) or prostaglandin A_2 (PGA_2). In contrast, the *gadd* genes were only strongly induced by medium depletion, MMS, or PGA_2. These and other studies indicate that there are probably multiple negative growth control mechanisms and that the *gadd* response differs from other negative growth control mechanisms. A working model is that the *gadd* response is activated by stresses that force cells out of cycle, while other negative growth control mechanisms are involved in controlling growth in cells programmed to enter G_0 such as during terminal differentiation or contact inhibition.

Recent studies indicate that the induction of *gadd* mRNA is by multiple mechanisms. By nuclear run-ons, medium depletion induces transcription. Both our group and N. Holbrook's group have recently found that *gadd45*-CAT and *gadd153*-CAT constructs can be induced by medium depletion when these constructs are stably integrated into chromosomal DNA. However, the increase in mRNA levels is also due to altered message stability. For example, J. Jackman has found that *gadd45* and *gadd34* mRNA stability in growth-arrested cells is increased; similar results have been found for *gadd153* N Holbrook's group.

Other stress-response genes, such as the heat shock protein genes, have also been studied. We have isolated a large collection of cDNA 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. 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. The search for additional DDI genes has involved collaborative efforts with several groups that have isolated genes involved in DNA repair. For example,

the expression of the newly-isolated x-ray DNA repair gene, *XRCC1*, has been studied in collaboration with L. Thompson. *XRCC1* was not DDI, but we did find decreased expression in growth-arrested cells. In collaboration with P. Doetsch, two genes with possible roles in DNA base damage repair have been studied: neither gene was DDI.

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 to alter the expression of these genes with recombinant vectors and antisense approaches. Antibodies to gadd45 protein will be developed. Studies will be continued on the expression of various DDI genes in mutant cells.

Publications

Fargnoli J, Holbrook NJ, and Fornace AJ Jr. Low-ratio hybridization subtraction, Analytical Biochemistry 1990;187:364-73.

Leuthy JD, Fargnoli J, Park JS, Fornace AJ Jr, Holbrook NJ. Isolation and characterization of the hamster *gadd153* gene: activation of promoter activity by agents that damage DNA, J Biol Chem 1990;265:16521-6.

Papathanasiou MA, Kerr N, Robbins JH, Mc Bride OW, Alamo I Jr, Barrett SF, Hickson I, Fornace AJ Jr. Induction by ionizing radiation of the *gadd45* gene in cultured human cells: lack of mediation by protein kinase C, Molec Cell Biol 1991,11:1009-16.

Nebert DW, Peterson DD, Fornace AJ Jr. Cellular responses to oxidative stress. In Hamilton GA, Reddy CC, eds. Biological Oxidation Systems. New York: Academic Press, 1990;69-84.

Nebert DW, Peterson DD, and Fornace AJ Jr. Cellular responses to oxidative stress: the [*Ah*] gene battery as a paradigm Environ Health Perspect 1990;88:13-25.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07186-02 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Other:	I. Alamo	Microbiologist	LMPH	NCI

COOPERATING UNITS *(if any)*

Applied Genetics, Freeport, New York (D. Yarosh).

LAB/BRANCH Laboratory of Molecular Pharmacology, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	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.)*

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 O-6 position of guanine but not at other sites in DNA. 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 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*. This has involved the production of recombinant O6AT protein and the development of antibodies using this recombinant protein.

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

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.

Large quantities of recombinant O6AT protein have been synthesized using bacterial expression vectors and our O6AT cDNA clone. This protein has been used to develop high affinity antibodies in collaboration with D. Yarosh.

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.

Publications

Fornace AJ Jr, Papathanasiou MA, Hollander MC, and Yarosh DB. Expression of the O^6-methylguanine-DNA methyltransferase *MGMT* gene in mer^+ and mer^- human tumor cells, Cancer Res 1990;50:7908-7911.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07187-02 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

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, DTP, DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

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. 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 *gadd153* RNA, this transcript was constitutively elevated in HN2-resistant derivatives of a human tumor cell line, Raji/HN2. 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, *gadd153*, and *gadd33*.

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

none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07188-02 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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.	Sr. 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:
0.05	0.05	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.)*

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.

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

With the expected departure of S. Wilson from the NIH, this project will be terminated at the end of this year. Some aspects of the DNA-damage responsiveness of ß-polymerase will be covered in project Z01 CM 07184-02.

Publications

Padmini PS, Lowy DR, Widen SG, Fornace AJ Jr, Wilson SH. Activation of transfected human ß-polymerase promoter by p21v-rasH expression, 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 07189-01 LMPH

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular, Cellular and Therapeutic Mechanisms in Amino Acid Deficiencies

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

PI: Marco Rabinovitz Research Chemist LMPH, NCI

COOPERATING UNITS *(if any)*

Dr. Jay R. Knutson, NHLBI, NIH

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP,DCT

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	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 activity of rabbit muscle phosphofructokinase was inhibited by transfer ribonucleic acid. This inhibition was reduced by inclusion of an aminoacyl-tRNA charging system. The results explain the loss of ATP in amino acid deprived cells, the significance of the critical role of fructose-1,6-diphosphate in peptide chain initiation, and other phenomena dependent upon this interaction between the protein synthetic and glycolytic systems.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Introduction

Attempts to reduce the rate of tumor growth in experimental animals by dietary restriction of a single, essential amino acid were initiated in the early days of experimental chemotherapy. The results were variable, but generally the tumor continued uninterrupted growth at the expense of the host, resulting in the premise that the tumor was a "nitrogen trap." At a later date interest was resumed by the observation that an antitumor agent in guinea pig serum was asparaginase, and many studies were initiated to evaluate the effect of amino acid limitation on therapy. In parallel studies in vitro observations were made that normal cells in culture rapidly ceased proliferation when subjected to an amino acid deficiency, while tumor or transformed cells continued to grow and died. This lead to studies with histidinol as an adjunct in therapy, since histidinol, the alcohol analog of the essential amino acid histidine, blocked histidine charging of transfer ribonucleic acid and protein synthesis, a situation that was lethal to tumor cells. Other investigators found that blocking amino acid activation for protein synthesis reduced both glucose utilization and ATP levels in cells. This caused polyribosome disaggregation in all cells studied except reticulocytes. These findings lead to the current investigation.

Objectives:

1) To understand the molecular lesions introduced in a cell when an amino acid is made unavailable for protein synthesis.

2) To define the initial molecular lesion and determine the cascade of biochemical events which are derivative of the initial lesion.

3) To determine where such lesions may be either amino acid or cell specific and how it may differ between normal proliferating cells and those of tumors.

4) To explore the various methods for introducing this lesion, such as elimination of an amino acid from a medium or ration, its degradation by an intracellular or extracellular enzyme, interference with its transport into the cell and inhibition of its activation for protein synthesis.

5) To determine how a block is protein synthesis by amino acid deprivation differs from blocks in peptide chain elongation and from production of aberrant, inactive proteins and enzymes synthesized by incorporation of selected amino acid antagonists.

6) Where applicable to investigate these events in purified enzyme systems, acellular lysate or organelle preparations, cells and tissues in culture and in the intact animal.

7) To apply the aforementioned information to the tumor-bearing animal in pilot studies, both as single agent therapy and as adjuvant to other agents.

Methods Employed:

1. Spectrophotometric assay of products of enzyme activity and its kinetic parameters.

2. Fluorimetric analysis of protein-nucleic acid interaction such as energy transfer and quenching.

Major Findings:

The key finding of this investigation is that uncharged transfer RNA (tRNA), which is known to accumulate in cells as a result of amino acid deprivation or action of amino acid analogs, directly inhibits the key controlling enzyme of glycolysis, phosphofructokinase. All further studies are directed toward expansion and development of this observation.

Proposed Course:

1. Inhibition of Phosphofructokinase by tRNA in Pure Enzyme Systems

Original observations will be extended by improved methodology, such as introduction of temperature control apparatus to our recording spectrophotometer and the use of tRNAs specific for individual amino acids. Preliminary evidence indicates that phenylalanine tRNA of yeast may serve such a purpose. This tRNA is also of interest since it contains a fluorescent base which will permit a method of interaction analysis independent of inhibition (see below). Also the use of valine tRNA will be investigated since the acylated form of this tRNA does not rapidly deacylate under the neutral conditions necessary for the assay of phosphofructokinase. It may thus be possible to study the inhibition of this enzyme with pure deacylated and acylated valine tRNA, thus obviating the necessity for inclusion of a charging system in the assay.

Detailed studies on the influence of various concentrations of nucleotides and fructose-6-phosphate will be followed, as well the effects of amino acid analogs which inhibit tRNA charging.

2. Intrinsic Protein and tRNA Fluorescence in Estimation of Phosphofructokinase Interaction with Transfer Ribonucleic Acid

The fluorescence of proteins is due principally to their content of tryptophan residues and the magnitude of this physical property has been used as a criterion for estimating the extent of protein-ligand interactions. Rabbit muscle phosphofructokinase has 10 tryptophan residues distributed throughout a molecule of 780 residues, making it a most suitable substrate for such a study. This method has already been used to evaluate the nature of binding of regulatory nucleotides to this enzyme. Phenylalanine tRNA contains a

fluorescent base (Ywye base) whose activity has been utilized in studying the interaction of this tRNA with the phenylalanine activating enzyme. Similar interactions with phosphofructokinase can be investigated with the use of both energy transfer and quenching parameters.

3. Inhibition of Phosphofructokinase by Amino Acid Deficiency in Cellular Systems

The decrease in the level of fructose-1,6-diphosphate in quiescent relative to proliferating cells has been noted for some time, however data indicating the rapidity with which this decrease takes place has not been evaluated. This requires a sudden block, such as that attainable by addition of an antagonist of amino acid activation rather than the removal of an amino acid from a complete nutrient medium. The residual nutrient within the cell as well as the availability of amino acids from protein turnover would preclude the latter analysis. It is proposed to estimate the kinetics of change of all hexose phosphates by standard spectrophotometric methods after a block in protein synthesis by inhibitors of amino acid activation. Both normal and tumor cells which are available in culture from other laboratories at the NIH will be studied.

Publications:

Rabinovitz M. Evidence for a role of phosphofructokinase and tRNA in the polyribosome disaggregation of amino acid deficiency, FEBS Lett, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07309-01 LMPH

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Analysis of the effects of stress and stress-response genes on the regulation of HIV-1 gene expression

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:	F. Carrier projected arrival: 8/12/91	Visiting Associate	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:
0.2	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.)*

This project will investigate the activation of HIV-1 gene expression by DNA-damaging agents and other stresses. Treatment of human cells with DNA-damaging agents, such as UV radiation, can lead to the activation of latent virus and to increased virus production. This activation is probably mediated by multiple mechanisms including the induction of a cellular Tat-like factor and the induction of at least one and probably more proteins that bind to specific elements in the HIV promoter. We plan to apply our expertise with DNA-damage-inducible (DDI) genes and their promoters to study the mechanisms involved in the induction of the HIV-1 promoter. One portion of this project will involve the use of various HIV promoter constructs fused to reporter genes such as chloramphenicol acetyltransferase (CAT). These constructs will be stably integrated in human cells and the effect of various deletions and mutations in the promoter will be analyzed. The effect of various inhibitors such as kinase inhibitors will be employed to delineate the role of different kinases and other proteins. Experiments such as band-shift assays will be employed to identify binding-factor elements; competition experiments with various sequences, which bind transcription factors, including novel DDI elements, which we have recently identified, will be undertaken to tentatively identify common factors. The effect of various types of DNA-damaging agents on induction will be studied; this may provide evidence for the mechanisms involved in induction and may also be of clinical importance when planning therapy for HIV-infected patients with malignancies.

Project Description

Objective: To study the DNA-damage-responses of the HIV-1 promoter and compare this to other DDI responses such as that of the *gadd* genes.

Methods Employed

Standard molecular biology techniques.

Major Findings

This project is funded by the NIH Intramural AIDS Targeted Antiviral Program, CAN 1 8322984 which was awarded in Dec., 1990. Initial efforts have been to recruit the necessary personnel and obtain necessary supplies and equipment.

Significance to Biomedical Research and the Program of the Institute

Elucidation of the mechanisms involved in HIV-1 induction by genotoxic stress may importance in understanding proviral activation and disease progression in AIDS. It may also be of clinical importance when planning therapy for HIV-infected patients with malignancies

Proposed Course

Studies are planned on regulation of the HIV gene expression by DNA-damaging agents. Herrlich's group has shown that the promoter of the HIV-1 long terminal repeat is strongly induced by DNA-damage; a substantial part of this effect is mediated by the transcription factor NFkB which is activated by protein kinase C. Investigations will be initiated to study activation of this HIV-1 gene by genotoxic stress and other stresses. Both PKC-dependent and PKC-independent mechanisms will be studied. The role of the protein products of our *gadd* genes in modulating HIV-1 expression are planned. The goal of these studies is to gain insight into the roles of stress, particularly genotoxic stress, in modulating HIV-1 gene expression and activating lytic viral infection.

Publications

none

ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The major objectives of the Laboratory of Medicinal Chemistry (LMC) are the discovery and development of new anticancer and anti-AIDS drugs. Because both cancer and AIDS patients often have complications associated with more conventional viral diseases, the LMC also evaluates the antiviral properties of newly synthesized compounds. Organic synthesis, analytical and polypeptide chemistry, computer-assisted molecular modeling, and biochemical pharmacology are the research areas emphasized to accomplish these goals.

Anticancer Drug Research

The fermentation product, neplanocin A, has provided the impetus for the preparation of synthetic analogues containing its unusual carbocyclic sugar. A practical result of this research effort is the cytidine analogue, CPE-C, which will begin a Phase I clinical trial this year. Several projects have been carried out to support this impending clinical work.

Cellular pharmacology studies indicate that CPE-C has an IC_{50} of 75nM in MOLT-4 cells. Intracellular CPE-CTP concentrations are about 50 times the plasma level, and the anabolite has a long half-life (9-14 hours). These cellular studies lead to the *in vivo* investigation of cytidine as a modulator of CPE-C toxicity. Cytidine was an effective rescue agent in mice with and without tumors. Cytidine administration at eight hours after CPE-C treatment allowed 2-3 times the LD_{90} doses of CPE-C to be given without killing the mice. While the antitumor activity was not enhanced in the presence of cytidine, neither was it diminished, suggesting that cytidine might play a safety role in CPE-C clinical studies.

The disposition and pharmacokinetics of CPE-C continuous infusion in dogs were studied in collaboration with the Toxicology Branch, DTP, in order to provide information for the INDA filing. An unexpected plasma *vs.* time profile was observed. In contrast to the situation in monkeys where it is the major metabolite, CPE-U was not found in dogs. Research into the enhancement of the current LMC analytical method for determination of CPE-C in biological fluids was prompted by the very low proposed clinical starting dose. A fluorescent derivative of CPE-C has been prepared which should allow quantitation to 0.01 μM in the Phase I studies.

A number of CPE-C analogues were synthesized, including 5-Aza-CPE-C. This compound inhibits CTP synthetase, but is much less potent than CPE-C. While replacement of the 4-amino group in cytidine with hydrogen (zebularine) resulted in a compound with antitumor, antiviral and enzyme inhibition properties, a similar change in CPE-C produces an inactive compound. Combining the structural features of neplanocin with those of another natural

product, psicofuranine, gave psicoplanocin A. This nucleoside, while non-cytotoxic in vitro, showed good activity against several viruses (RSV, measles, parainfluenza). 3-Deazaneplanocin A, synthesized several years ago by the LMC, continues to show activity in additional antiviral tests (African swine flu and measles in vitro, and Ebola in vivo).

2-Oxopyrimidine riboside (zebularine, Zeb) possesses an unusual combination of biological properties. In vivo, zebularine and its 5-fluoro analogue have very good activity against L1210 leukemia. In vitro, Zeb activity has been observed against several viruses. This compound also is a potent inhibitor of cytidine deaminase. Utilizing NMR spectroscopy, the base-catalyzed decomposition of Zeb was elucidated in an attempt to understand the mechanism of antitumor activity in this series. Aglycon ring-opening, followed by decomposition to malonaldehyde and an unusual bicyclic compound was observed. Utilizing the 5-fluoro analogue, a more stable aldehydic intermediate was produced which facilitated the mechanistic interpretation. If Zeb or its 5-fluoro analogue were incorporated into nucleic acids prior to decomposition, reactive malonaldehyde could be released at a defined site providing an unusual mechanism of antitumor action for a nucleoside.

Protein kinase C (PKC) plays an important role in initiating a number of cancer-related biochemical events. In an attempt to define the diacylglycerol (DAG) stereochemical parameters important for the activation of PKC, eight γ-lactones were synthesized and tested in a phorbol ester displacement assay. These rigid DAG analogues have yielded a compound as potent as DAG and have provided important data relative to DAG geometry and the optimal nature of the fatty acid side-chain.

A second protein kinase project relates to the synthesis and evaluation of inhibitors of tyrosine kinases (TK) which could modulate cellular signal transduction events important in cancer processes. Evaluation of 13 opiates showed that phenazocine inhibited the autophosphorylation of the EGF receptor with a potency similar to that of erbstatin, a standard inhibitor. Computer-assisted molecular modeling and quantitative structure-activity studies are being used to guide the synthesis of a second class of inhibitors, polyhydroxylated cinnamamide derivatives. Platelet-derived growth factor and the insulin receptor are among the evaluation systems used.

A hydrolytically stable analogue of O-phosphotyrosine has been synthesized for use as a mimetic in peptides which might prevent the action of TK by interfering with the recognition of endogenous substrates. Because of its unique combination of protecting groups, this phosphonate-containing amino acid is suitable for automated solid-phase peptide synthesis.

Laminin is an extracellular protein implicated in metastasis. Using the sense/antisense concept of protein molecular recognition, the design and synthesis of multiple polypeptides to various regions of laminin has been undertaken.

Using a powerful, dedicated computer system, molecular modeling has been employed to assist LMC synthetic chemists design and prioritize target compounds as well as to provide basic information regarding the conformational

effects of solvation on molecules in solution. These studies have had impact on both the PKC and TK inhibitor projects mentioned above, as well as synthetic nucleoside work. Effort to convert the 2-dimensional DTP database (DIS) to 3-dimensional atomic coordinates has resulted in 3-D coordinates for 125,000 unrestricted compounds. Generation of the remainder of the 3-D structural database, which consists mostly of discreet materials, awaits the appropriate software for the DTP VAX at FDRDC.

Anti-AIDS Drug Research

Inosine monophosphate plays an important role in the anti-HIV activity of both dideoxyinosine (ddI) and dideoxyguanosine (ddG). It is the phosphate donor in the first step of the biochemical activation process which leads ultimately to ddATP, the anti-HIV-active form of the drug. It was found that the coadministration of ribavirin, an inosine monophosphate dehydrogenase (IMPD) inhibitor, made ddI four times more potent against HIV by increasing the amount of IMP available for the transphosphorylation reaction.

Hepatitis B viral (HBV) infections are responsive to dideoxynucleosides (ddN) in vitro, but the relationship between structure and activity is different than that observed in HIV experiments. In an attempt to resolve this difference, a study of the phosphorylation of ddN in hepatocytes was conducted to determine whether anti-HBV activity was related to 5′-phosphate generation in liver cells. The amount of phosphorylation did not correlate with anti-HBV activity suggesting that the optimal activity of ddG in this system may be related to the interference of terminal dG incorporation in viral DNA production.

Two dideoxycytidine (ddC) metabolites have been isolated from MOLT-4 cells and identified as ddCDP-choline and ddCDP-ethanolamine by mass spectral comparison with synthetically prepared material. Whether these compounds are implicated in the peripheral neuropathy observed with ddC remains to be determined.

ddI is very unstable under acidic conditions, making oral administration somewhat difficult. Clinical pharmacology studies supporting the COP clinical trial with ddI have shown that various oral formulations utilizing antacids, buffers and enteric coated preparations provide 20-40% ddI bioavailability. Ranitidine and ganciclovir, drugs often used in AIDS patients, affect neither the bioavailibility nor the pharmacokinetics of ddI.

The LMC approach to solving the dideoxypurine nucleoside (ddN) acid-instability problem has been the synthesis of ddN containing a 2′-fluoro substituent. This tactic makes these materials stable not only to acidic conditions, but also to the action of purine nucleoside phosphorylase (PNP), an enzyme which degrades ddI. A new method for the synthesis of 2′-fluoro ddN derivatives has been developed which greatly simplifies the preparation of dideoxy analogues in this series. Using this synthetic method, six purines and seven pyrimidines were prepared and evaluated by DTP and COP using CEM and ATH8, respectively, as host cells for HIV. Among the pyrimidines, the uridine and thymidine analogues were without activity, while most of the cytidine

analogues were active. This finding probably can be ascribed to the fact that different kinases are utilized to activate these two types of pyrimidine dideoxynucleosides.

The 2′-F-dideoxypurines synthesized proved to have unusual and potentially useful properties. Adenosine deaminase (ADA) was found to convert inactive 6-substituted purine ddNs to the anti-HIV-active compound, 2′-F-ddI. This result indicates that the 6-substituted analogues are prodrugs of 2′-F-ddI. Since the transport properties of these lipophilic compounds are different from 2′-F-ddI, they have the potential for enhanced treatment of HIV in biological sanctuaries, eg. central nervous system (CNS). This concept could be of clinical importance since ADA levels are very high in humans.

The ability of these 6-substituted purine compounds to act as substrates for ADA was quantified by the determination of K_M and V_{max} values as well as by the measurement of relative rates of deamination. Efforts are underway to predict the rate of mammalian ADA conversion *in vivo* from the more easily measured *in vitro* data.

An analytical method was developed and used to measure 2′-F-ddA/I plasma and cerebrospinal fluid (CSF) pharmacokinetics in the rhesus monkey. Plasma elimination is biexponential with a terminal $t_{1/2}$ of 14 minutes. The CSF to plasma ratio of 2′-F-ddA (measured as 2′-F-ddI) was 9-14% which is greater than ddI (4%) and ddC (3%) but less than AZT (21%).

A rapid, microscale method has been developed and used to determine octanol-water partition coefficients for over 80 nucleosides of potential interest in AIDS and cancer chemotherapy. The initial use of these values is in attempts to predict drug penetration into the CNS.

Using the anti-HIV fermentation product oxetanocin as a model, the first dideoxyapiose nucleosides were synthesized. A *cis* isomer is active and appears to be an interesting lead. Carbocyclic members of the ddN series are also in preparation. Computer-assisted molecular modeling has been used effectively in designing these molecules for synthesis.

Over 50 polypeptides have been designed and synthesized as inhibitors to various regions of HIV protease using the sense/antisense concept. Tests against the isolated enzyme revealed that an 11-mer designed to react with the flap region was the best inhibitor (IC_{50} = 160 μM). The all D-isomer was also active but only one-third as potent. Additional modifications decreased inhibitory activity. These compounds were not active in the DTP *in vitro* anti-HIV screen.

Continuous-flow fast atom bombardment mass spectrometry combined with on-line capillary liquid chromatography (FAB/LC/MS) is being investigated as a powerful tool for the separation and identification of mixtures difficult to analyze by other methods. Non-volatile compounds are amenable to this separation and identification technique and, for this reason, nucleotides are a particular focus of initial investigations. A 65-fold enhancement of sensitivity for nucleotide diphosphate conjugates can be achieved utilizing anion-exchange micro-column LC with FAB mass spectrometry. Further

enhancements of sensitivity were found when quaternary amine surfactants were utilized in the elution solvent.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06173-06 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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
Other:	John S. Driscoll	Chief	LMC
	Greg Buenger	IRTA Fellow	LMC
	Joseph J. Barchi, Jr.	Senior Staff Fellow	LMC
	Ronald J. Wysocki, Jr.	IRTA Fellow	LMC
	Maqbool A. Siddiqui	Chemist	LMC

COOPERATING UNITS *(if any)*
Toxicology Branch, DTP, DCT, NCI
Clinical Oncology Program, DCT, NCI

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 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.)*

The new and more expedient method developed for the synthesis of 2'-fluoro-β-D-threo-dideoxy nucleosides was successfully extended to a series of new purine and pyrimidine analogues. Among the new pyrimidine analogues prepared, 1-(2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl)cytosine and its 5-fluorocytosine analogue displayed good anti-HIV activity. None of the uracil derivatives were active. Among the purines, 9-(2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl)-6-chloropurine behaved as a prodrug of the active hypoxanthine nucleoside after activation by adenosine deaminase. Finally, the weak anti-HIV activity of 9-(2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl)guanosine was increased significantly when used in combination with inosine monophosphate dehydrogenase inhibitors such as tiazofurin or ribavirin.

A number of ring-enlarged analogues of the anti-HIV-active fermentation product oxetanocin A have been synthesized with the intent of assessing the importance of the extra hydroxymethyl side chain to the anti-HIV activity. Some of the resulting dideoxyapiose and carbocyclic nucleosides made represent new chemical structures. Their biological evaluation is in progress.

6-Substituted-2'-fluoro-dideoxy purine nucleosides which are substrates for adenosine deaminase (ADA) are being synthesized as prodrug forms of F-ddI and F-ddG. The in vitro anti-HIV activities of the 6-chloro and N6-methylamino analogues were found to be abolished by the ADA inhibitor, 2'-deoxycoformycin, and augmented by the addition of ADA to the culture medium. These prodrugs are being designed as lipophilic molecules with potential for transport to HIV sanctuaries, such as the CNS, prior to activation by ADA.

Project Description:

General Objective: The objective of this project is the discovery of effective and stable 2′,3′-dideoxynucleosides 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 "pro-drug" purine dideoxynucleoside analogues activated by adenosine deaminase (ADA).

Major Findings:

Synthesis of Acid-Stable Dideoxypurine and Pyrimidine Nucleosides (Dr. Marquez, Mr. Siddiqui, Dr. Barchi, Dr. Wysocki, Dr. Buenger, Dr. Driscoll): A more extensive study of 2′-F-β-D-threo-dideoxy purine and pyrimidine nucleosides was undertaken as a result of the good and reproducible anti-HIV activity displayed by the parent compounds 1 and 2. Compounds synthesized in the purine series included analogues with 6-chloropurine (3), 2-amino-6-chloropurine (4), guanine (5), and 2,6-diaminopurine (6, in progress) aglycons, while in the pyrimidine series it included cytosine (7), 5-fluorocytosine (8), 5-chlorocytosine (9), uracil (10), 5-fluorouracil (11), 5-chlorouracil (12), and thymine (13) aglycons. All of these compounds were prepared via a new approach that employed the fluorodideoxy sugar intermediate 14. Use of this intermediate, which was developed in our laboratory, greatly simplified the synthesis of all these compounds. The presence of the fluorine atom in these molecules endows them with almost complete chemical and enzymatic stability towards cleavage of the glycosilic bond. The effect of fluorine on the anti-HIV activity of these compounds was assessed. Compounds 7 and 8 were found active in the DTP anti-HIV testing program and compound 5, although less potent than 1, was found to be effective in the presence of inosine monophophate dehydrogenase inhibitors (i.e., ribavirin, tiazofurin). Compound 3 is also of interest because it behaves as a prodrug of 2 after hydrolysis by ADA. None of the uracil derivatives (10-13) showed anti-HIV activity.

Synthesis of Sugar-Modified Dideoxynucleoside Analogues (Dr. Wysocki, Dr. Buenger, Dr. Marquez): Encouraged by the activity of 15, a ring-enlarged analogue of the active fermentation product oxetanocin A (21), a group of compounds was synthesized (16-20) in which the number and position of the hydroxymethyl side chains was varied. Two of these compounds (16 and 20) are the first known examples of dideoxyapiose nucleosides. Since in some of these compounds the α-anomer is equivalent to a dideoxynucleoside in which the O-4′ oxygen and the C-2′ carbon are interchanged (ie. 15 vs 18), the synthesis of the carbocyclic dideoxynucleoside analogue 22 was also undertaken. Due to symmetry, the α- and β-anomers of compound 22 are the same compound. To complete this series only the synthesis of compound 19 is still pending.

Biological evaluation is in progress, although preliminary indications are that compound 16 is inactive.

Synthesis of "Pro-Drug" Purine Dideoxynucleosides Analogues Activated by Adenosine Deaminase (ADA)(Dr. Driscoll, Mr. Siddiqui, Dr. Barchi, Dr. Marquez): An investigation into the mechanism of anti-HIV action of certain 6-substituted dideoxypurine nucleosides (ddN) revealed that added deoxycoformycin abolished activity while added adenosine deaminase (ADA) augmented it. Since ADA is present in such large amounts in humans, the observation mentioned above suggested the design of F-ddI and F-ddG prodrugs which could have transport properties which facilitate entry into sanctuaries such as the CNS, prior to conversion to the parent drug by ADA. Toxicity and formulation properties of these prodrugs might also be different relative to the parent drug. The synthesis and activity of 2′-F-dideoxypurine nucleosides containing 6-chloro and N6-methylamino groups indicate the utility of this concept. Additional new 6-substituted ddN, capable of ADA activation, are being synthesized.

Publications:

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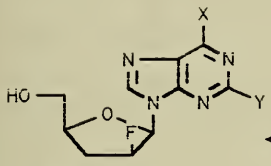
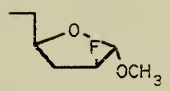


1, X=NH_2, Y=H
2, X=OH, Y=H
3, X=Cl, Y=H
4, X=Cl, Y=NH_2
5, X=OH, Y=NH_2
6, X=NH_2, Y=NH_2



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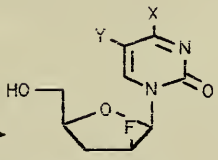
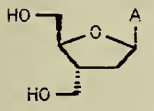


7, X=NH_2, Y=H
8, X=NH_2, Y=F
9, X=NH_2, Y=Cl
10, X=OH, Y=H
11, X=OH, Y=F
12, X=OH, Y=Cl
13, X=OH, Y=CH_3



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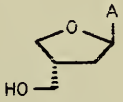

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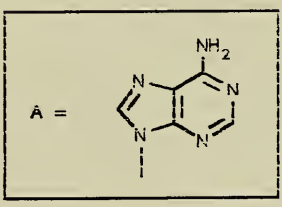
17 (ddA)



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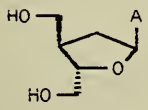

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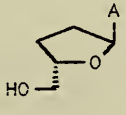

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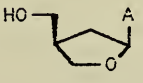

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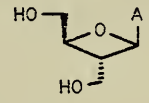

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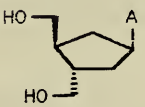


22

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06174-06 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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
Other:	John S. Driscoll	Chief	LMC
	Michael Bodenteich	Visiting Fellow	LMC
	Greg Buenger	IRTA Fellow	LMC
	Benjamin B. Lim	IRTA Fellow	LMC
	Pamela L. Russ	Chemist	LMC

COOPERATING UNITS *(if any)*
Southern Research Institute, Birmingham, Alabama
USAMRIID, Fort Detrick, Maryland
Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 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.)*

Among the new cyclopentenyl nucleosides synthesized and evaluated as antitumor and antiviral agents, 5-aza-cyclopentenylcytosine (5-aza-CPE-C) and the 2-oxopyrimidine analogue (CPE-Z) displayed no biological activity. In the purine series, the carbocyclic psicose analogue, psicoplanocin A, had marked activity against RSV, measles, and parainfluenza viruses. This activity is consistent with a mechanism of action similar to that of neplanocin A.

The synthesis of (-)-carbodine, an antitumor cyclopentyl nucleoside previously reported in the literature as a racemate, was achieved from cyclopentenyl cytosine (CPE-C). The isomeric compound (-)-isocarbodine was also synthesized. (-)-Carbodine was ca. twofold more potent than the racemate. (-)-Isocarbodine was unexpectedly effective against human influenza virus.

Several new cyclopentenyl nucleosides inspired by two natural products neplanocin A and clitocine, have been synthesized. Their biological evaluation is in progress.

The synthesis of the cyclopentenyl nucleoside analogue of the antitumor drug tiazofurin is near completion.

3-Deazaneplanocin A has shown potent activity in vitro against the African swine flu virus, as well as synergistic activity with ribavirin against the measles virus. This compound is also active in vivo against the Ebola virus in the SCID mouse model.

Project Description:

Ojective: The objective of this work is to accomplish the synthesis of specific cyclopentenyl carbocyclic nucleoside analogues and to investigate their properties 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. Buenger, Dr. Marquez): The cyclopentenyl carbocyclic nucleoside analogue of 5-azacytidine (2, 5-aza-CPE-C) was synthesized and evaluated for antitumor and antiviral activity. Despite its structural similarity with the biologically active cyclopentenyl cytosine [CPE-C (1)], 5-aza-CPE-C was between 100 to 1000 times less cytotoxic than CPE-C to L1210 and Molt 4 cells. Its capacitiy to lower CTP pools in the same cells also was correspondingly lower. As an antiviral agent, 2 had the same spectrum of activity as CPE-C but with much lower potency. The corresponding CPE-2-oxo-pyrimidine nucleoside (3, CPE-Z) was also synthesized. Preliminary biological results indicated that the compound was neither cytotoxic (in vitro L1210) nor inhibitory to the enzyme cytidine deaminase.

Carbocyclic Nucleoside Analogues Derived From a Modified Cyclopentenyl Moiety (Dr. Bodenteich, Ms. Ross, Dr. Marquez): The biological evaluation of the first carbocyclic ketosugar nucleoside (psicoplanocin A, 6) revealed no cytoxicity toward L1210 cells in vitro. The compound, however, had marked activity against respiratory syncytial virus, measles, and parainfluenza. Although the compound was 10 to 25 times less potent than neplanocin A (4) against the same viruses, it was also less cytotoxic. The selectivity indices were the same for both drugs. The syntheses of (-)-carbodine (7) and (-)-isocarbodine (8) was completed. (-)-Carbodine was a more potent antitumor and antiviral agent relative to its racemate. An interesting finding was the unexpected marked activity displayed by isocarbodine against human influenza virus (viral rating = 2.6).

CPE-Purine Analogues (Dr. Lim, Dr. Marquez): Both neplanocin A (4) and the LMC's 3-deazaneplanocin A, 5, possess significant antitumor activity in addition to their recognized antiviral utility. A new series of compounds (10-13), inspired by neplanocin A and in the unusual cytotoxic natural product clitocine (9), have been synthesized. Biological evaluation is in progress.

CPE-Tiazofurin (Dr. Bodenteich, Dr. Marquez): Tiazofurin (14) is a compound that has shown therapeutic activity in end-stage acute nonlymphocytic leukemia and blast crisis of chronic granulocytic leukemia. The cyclopentenyl analogue (CPE-T, 15) was selected as a target and structure 16 represents the most forward progress made to date.

3-Deazaneplanocin A (Dr. Marquez, Dr. Driscoll): New antiviral activities continue to be discovered for 3-DN, the most potent known inhibitor os S-adenosylhomocysteine hydrolase. 3-DN is a very potent inhibitor of African swine flu virus possessing a selectivity index of 5000 (De Clercq, Rega

Institute). The compound is also reported to be a potent in vivo inhibitor of the Ebola virus in the SCID mouse (U.S. Army). Synergistic effects against the measles virus in vitro have been noted for the combination of 3-DN with ribavirin (Baylor College of Medicine).

Publications:

Kim SK, Fuller RW, Marquez VE. Synthesis and Biological Activity of Ara and 2'-Deoxycyclopentenyl Cytosine Nucleoside Analogues, Nucleosides Nucleotides 1990;9:663-677.

Copp RR, Marquez VE. Synthesis of Two Cyclopentenyl-3-Deazapyrimidine Carbocyclic Nucleosides Related to Cytidine and Uridine, J Med Chem 1990;34: 208-212.

Marquez VE, Bodenteich M, Copp RR, Lim BB: Synthesis and Biological Activity of Novel Carbocyclic Nucleosides, Nucl Acids Res Symposium Ser No. 22, 1990; 35-36.

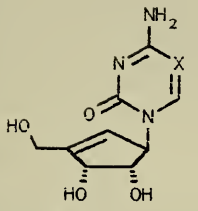
Bodenteich M, Marquez VE. Synthesis of (+/-)-Psicoplanocin A, Tetrahedron Lett 1990;31:5977-5980.

Marquez VE, Bodenteich M. Psicoplanocin A. A Synthetic Carbocyclic Nucleoside with the Combined Features of Neplanocin A and Psicofuranine, Nucleosides Nucleotides, 1991;10:311-314.

De Clercq E, Murase J, Marquez, VE. Broad-Spectrum Antiviral and Cytocidal Activity of Cyclopentenylcytosine, a Carbocyclic Nucleoside Targeted at CTP Synthetase, Biochem Pharmacol 1991;41:1821-1829.

Marquez VE, Lim MI, Khan MS, Kaskar B. (4R,5R)-(-)-3-Benzyloxymethyl-4,5-O-isopropylidene-2-cyclopentenone. An optically Active α,β-Unsaturated Cyclopentenone for the Synthesis of Neplanocin A and other Cyclopentene Carbocyclic Nucleosides. In: Townsend LB and Tipson RS, eds. Nucleic Acid Chemistry. Improved and New Synthetic Procedures Methods and Techniques. Part 4., New York: Wiley, 1991;27-35.

Marquez VE, Lim MI, 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 4., New York: Wiley, 1991;251-260.



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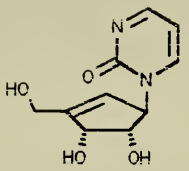

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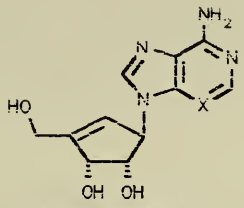

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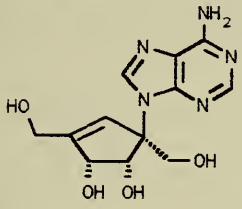
5, X=CH



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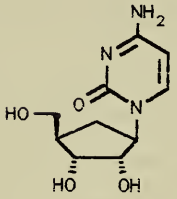

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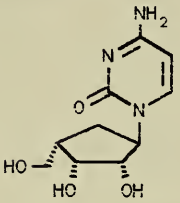

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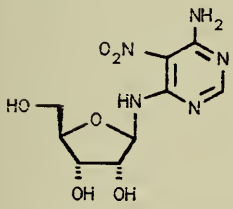

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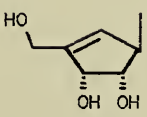

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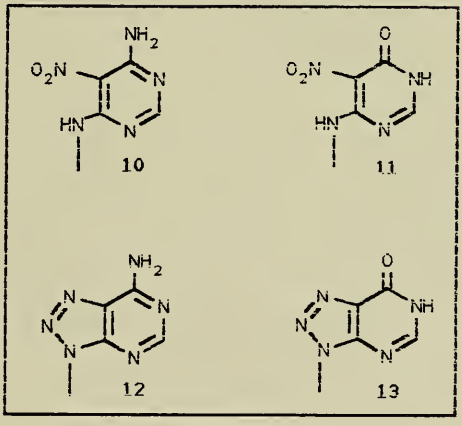

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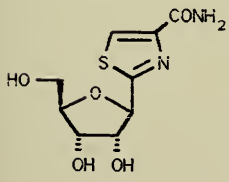

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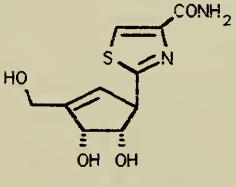

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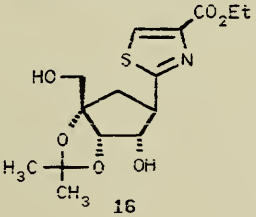


16

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06175-06 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS *(if any)*

Michigan Cancer Foundation, Detroit, Michigan

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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 project continued for this year at a reduced level of activity. In collaboration with Dr. Judith Christman of the Michigan Cancer Foundation the ability of our synthetic oligodeoxynucleotides to function as substrates for DNA methylases was studied. It was found that differences in substrate properties were related to the position of the modified base dihydro-5-azacytosine. It is expected that conversion of the dihydro-5-azacytosine moiety to the aromatic 5-azacytosine base will change these oligodeoxynucleotides into effective inhibitors of the enzyme. Oxidation of the dihydro-5-azacytosine moiety in these 26-mer oligodeoxynucleotides continues to be difficult to monitor due to the instability towards alkali shown by both types of oligomers. Assessment of their differences by biological activity might be the only way to monitor the success of the oxidation reaction.

Project Description:

Objective: The objective of this work is to prepare synthetic oligonucleotides containing 5-azacytosine (AC), or its reduced 5,6-dihydro-5-azacytosine (DHAC) analogue, in specific positions of the oligomer. The resulting oligonucleotides are being studied as DNA methylase substrate/inhibitors.

Major Findings:

DHAC-modified 26-mers as substrates for DNA Methylase (Dr. Marquez): Preliminary studies with the parent oligodeoxynucleotide (5'-CCGGCCATTACGGATCCGTCCTGGGC-3) and the DHAC-11- and DHAC-17-substituted oligomers, indicated that all three oligodeoxynucleotides were good substrates for DNA methylase. However, the DHAC-11-substituted oligodeoxynucleotide was a superior substrate (2-fold better) than either the parent or the DHAC-17-substitued oligodeoxynucleotide. No inhibition of DNA methylase was detected.

AC-modified 26-mers as substrates for DNA Methylase (Dr. Marquez): Reaction conditions that were developed for the successful oxidation of the DHACpT dimer were tried for the DHAC-17 oligodeoxynucleotide. Since the instability of the resulting AC moiety under alkaline conditions was supposed to be diagnostic of a successful oxidation reaction, the stability of the modified DHAC-17 oligo was studied. Unfortunately, both starting and oxidized oligodeoxynucleotides are extremely sensitive to alkaline conditions. This instability prevents a conclusive assessment of the success of the oxidation reaction by chemical means. Biological evaluation of the oxidized DHAC-17 oligo with DNA methylase will be performed to determine if the oligomer could be converted from a substrate to an inhibitor due to the presence of the AC moiety. These studies are in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06176-06 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Other:	John S. Driscoll	Chief	LMC
	Joseph J. Barchi, Jr.	Senior Staff Fellow	LMC
	Kelly Teng	IRTA Fellow	LMC
	Jeewoo Lee	Visiting Fellow	LMC

COOPERATING UNITS *(if any)*

Laboratory of Cellular Carcinogenesis of Tumor Promotion, DCE, NCI

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.50	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 mechanism of the base-catalyzed decomposition of zebularine and similar 2-oxopyrimidine nucleosides has been completely elucidated. The previously elusive electrophilic species generated during the decomposition of the drug(s) has been identified as malonaldehyde. Malonaldehyde is a very reactive species capable of interacting with nucleic acids. The relevance of this phenomenon to the antitumor effect of zebularine and its analogues is under investigation.

Some unique five-membered ring γ-lactones substituted with either myristic or oleic acid ester groups were found to be biologically equivalent to the endogenous protein kinase-C (PK-C) agonist diacylglycerol (DAG). These compounds have a fixed conformation which is presumed to be similar to the conformation adopted by DAG when binding to PK-C. Two long alkyl-ether analogues of these γ-lactones behaved also as good DAG surrogates. These compounds have the advantage of being hydrolytically stable. Based on these leads, a second generation of rigid DAG analogues was synthesized. Biological evaluation of these new compounds is in progress.

Project Description:

Objective: The objective of this project is to design new biologically active molecules and to study their mode of action as mechanism-based enzyme activators/inhibitors. Some of these molecules are per se potential antitumor or antiviral agents, while others constitute probes designed to elucidate drug-receptor interactions.

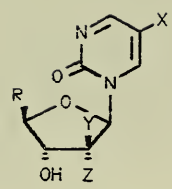
Major Findings:

Pyrimidine-2-one Nucleosides. A Class of Cytidine/dCMP Deaminase Inhibitors with Unusual Chemical and Biological Properties (Dr. Barchi, Dr. Marquez): The mechanism of the base-catalyzed decomposition of 2-oxo-pyrimidine ribosides (1-4) has been completely elucidated. The initial step is the reversible ring-opening of the pyrimidine ring to the aldehyde intermediate 7. This intermediate can either reclose back to the starting material or decompose via loss of a fragment that rapidly hydrolyzes to malonaldehyde. The remaining portion of the molecule rearranges to a cyclic carbamate sugar (8). The malonaldehyde produced was identified by its convertion to the known pyrazole derivative 9. The identical results obtained with compounds lacking the 5'-OH (2 and 3) ruled out an intermediate $O^{5'}$-C_6 cyclonucleoside in this rearrangement. The relevance of this mechanism to the antitumor properties of these compounds is under investigation. In other related aspects, the synthesis of the 2'-deoxy- and 2'-F-2'-deoxy- analogues (5 and 6) was completed. These compounds are expected to function as potential inhibitors dCMP deaminase after metabolic conversion to the monophosphate.

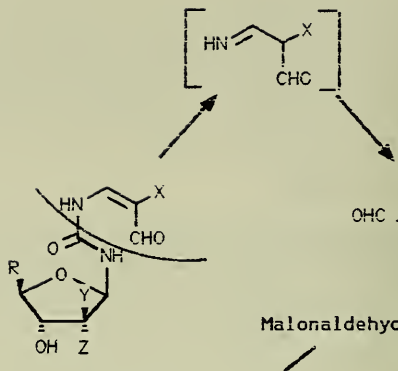
Synthesis of Protein Kinase C (PK-C) Inhibitors (Dr. Teng, Dr. Lee, Dr. Marquez): A unique five-membered ring γ-lactone (10) was one of eight possible isomers synthesized as conformationally rigid analogues of diacylglycerol (DAG). Results from the inhibition of phorbol ester binding, PK-C activation, and inhibition of epidermal growth factor (EGF) receptor, indicated that this lactone was biologically equivalent to DAG. Changing the nature of the fatty acid ester in 10 identified the oleate side chain as the one yielding the most potent analogue (11). Contrary to DAG, however, the difference in biological potency observed for lactone 10 and its optical antipode was less than that reported between DAG and its enantiomer. The same appears to be true for the corresponding ether analogues (12 and 13): K_i 3.31 vs 4.64 in the phorbol binding assay. Target compounds 14 and 15, in which the lipohilic side chain originates at the C-2 carbon of the lactone, have been synthesized. Their biological evaluation has not yet been completed. Finally, synthesis of a second type of rigid DAG analogue, in which the glycerol backbone spans across a bicyclic system (16), is near completion. Structure 17 represents the most forward progress.

Publications:

Goldstein BM, Leary JF, Farley BA, Marquez VE, Levy PC, Rowley PT: Induction of HL60 Cell Differentiation by Tiazofurin and its analogues: Characterization and and Efficacy, Blood. in press

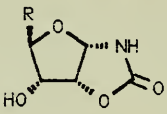


1, X=Y=H, Z=OH, R=CH_2OH
2, X=Y=H, Z=OH, R=CH_2OCH_3
3, X=Y=H, Z=OH, R=CH_3
4, X=F, Y=H, Z=OH, R=CH_2OH
5, X=Y=Z=H, R=CH_2OH
6, X=Z=H, Y=F, R=CH_2OH



Malonaldehyde (X=H)

7



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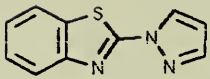
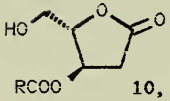
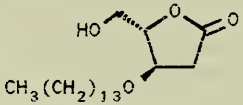


9

R = CH_3 or OCH_3 or CH_2OH



10, R=$(CH_2)_{12}CH_3$
11, R=$(CH_2)_7C{=}C(CH_2)_7CH_3$



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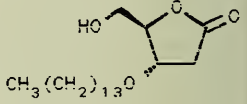

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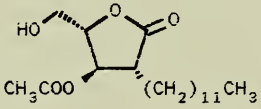

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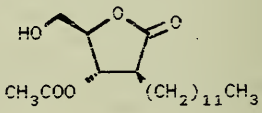

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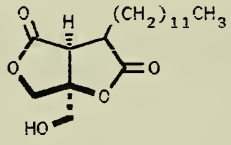

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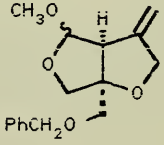


17

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06198-02 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Other: V. E. Marquez	Deputy Chief	LMC
B. B. Lim	IRTA Fellow	LMC
P. L. Russ	Chemist	LMC
M. C. Nicklaus	Visiting Fellow	LMC

COOPERATING UNITS *(if any)*

LTVB, DCE, NCI; CPB, COP, DCT, NCI; Howard Hughes Medical Institute, UCSF, San Francisco, CA; Joslin Diabetes Research Center, Boston, MA

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	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.)*

Several approaches are being taken to develop agents which modulate cellular O-phosphotyrosine-utilizing signal transduction processes. In one study, which examined the possible inhibition of protein-tyrosine kinases (PTK) by opiates, it was found that (+/-)-phenazocine (1) was a potent inhibitor of epidermal growth factor receptor (EGFR) PTK (IC_{50} = 12 μM) with an activity approaching that of erbstatin (IC_{50} = 5 μM). (+/-)-Phenazocine represents a potentially important lead in the development of a new class of PTK inhibitors. In a separate study, a series of phosphonate-containing styrenes (2) was found to be inactive as inhibitors of either EGFR or $p56^{lck}$ PTKs. Additional phosphonate-containing analogues of (4) are being prepared to more fully define the potential of phosphonate-bearing PTK inhibitors. In another investigation, a series of hydroxylated cyano-cinnamamides (5) were prepared and examined for their ability to inhibit various PTKs ($p56^{lck}$, platelet derived growth factor (PDGF) and insulin receptor). Several analogues exhibited markedly different potencies among the different PTKs, a fact which is being analyzed by computer-generated molecular modeling to determine structural criteria for kinase selectivity and binding to the catalytic site. A series of conformationally constrained analogues (6) is being prepared to further elaborate these results. Finally, a compound, 7, has been prepared as a hydrolytically stable mimetic of O-phosphotyrosine which is suitably protected for incorporation into peptides using solid-phase techniques. Compound 7 is being used to construct mimetics of O-phosphotyrosine-containing peptides which are intended as inhibitors of PTK substrate recognition and of O-phosphotyrosine phosphatases (which are involved in PTK modulation).

Project Description:

General Objective:

The primary objective of this project is the development of agents which modulate the cellular O-phosphotyrosine-utilizing signal transduction processes. Such compounds have potential use as biochemical tools for investigating signal transduction mechanisms and as therapeutic agents for the treatment of cancer and various other disorders, such as diabetes.

Specific Objectives:

1) The development of protein-tyrosine kinase (PTK) inhibitors which function by competitively inhibiting the binding of tyrosyl-containing substrate to the kinase catalytic site. The following structural motifs are being utilized: a) opioid analogues; b) phosphonate-containing analogues. c) polyhydroxy aromatic analogues.
2) The development of phosphonate-containing peptides and peptide mimetics which inhibit the function of PTKs by interfering either with the recognition of endogenous substrate or through the inhibition of O-phosphotyrosine phosphatases which constitutively regulate the action of PTKs.

Major Findings:

Opioid-Based Inhibitors (Dr. Burke, Dr. Marquez):
A total of 13 opiates, representing examples of the major classes of opioid nuclei, were assayed for inhibition of autophosphorylation of epidermal growth factor receptor (EGFR) tyrosine kinase. Only (+/-)-phenazocine (**1**) exhibited significant inhibition (IC_{50} = 12 μM) with an activity in the range of the potent tyrosine kinase inhibitor erbstatin (IC_{50} = 5 μM). Structurally, (+/-)-phenazocine potentially represents an important lead in the design of a new class of PTK inhibitors.

Phosphonate-Containing Inhibitors (Dr. Burke, Ms. Russ, Dr. Lim, Dr. Marquez):
A series of phosphonate-containing benzylidenes (**2**) was prepared as well as naphthalene phosphonates. With the exception of naphthalene phosphonate **3** (EGFR; IC_{50} = 250 μM), none of the compounds exhibited significant inhibition of autophosphorylation (EGFR and $p56^{lck}$) or transphosphorylation ($p56^{lck}$). A further structure-activity study is in progress to examine in more detail the interaction of aryl-phosphonates (**4**) with PTKs.

Polyhydroxylated Aromatic Inhibitors (Dr. Burke, Dr. Lim, Dr. Nicklaus, Dr. Marquez):
A series of polyhydroxylated cyano-cinnamamide derivatives (**5**) have been prepared and their inhibitory activities examined in several different PTK systems ($p56^{lck}$, Platelet-Derived Growth Factor Receptor (PDGFR) and insulin receptor). Differences in potency for individual analogues among the three kinase systems are being correlated with the aryl-substitution pattern using

computer-generated molecular modeling. Results of these studies are intended to define which structural parameters confer inhibitor specificity among the various kinases and to indirectly probe enzymatic features within the catalytic binding site. Bicyclic analogues (6) are also being prepared as conformationally constrained analogues which could potentially afford new structural classes of PTK inhibitors.

Inhibition of Substrate Recognition (Dr. Burke, Ms. Russ, Dr. Marquez): Compound **7** has been prepared as a hydrolytically stable analogue of O-phosphotyrosine which is suitably protected for incorporation into peptides using standard solid-phase techniques. Multiple studies are in progress to utilize this derivative in the preparation of peptides as mimetics of the corresponding O-phosphotyrosyl-containing peptides. These peptides have potential use as phosphatase inhibitors and as compounds which prevent the action of PTKs by interfering with the recognition of endogenous substrates.

Publications:

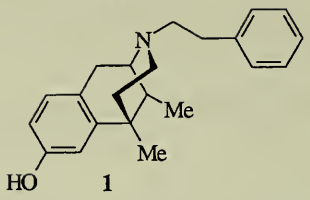
Burke TR Jr. A direct approach toward the synthesis of analogs of erbstatin, Org Prep Proc Int 1991;23:127-130.

Burke TR Jr, Li Z-H, Bolen JB, Chapekar M, Gang Y, Glazer RI, Rice KC and Marquez VE. Examination of the possible mediation of antineoplastic effects of opiates through the inhibition of tyrosine-specific protein kinase, Biochem Pharm 1991;41:R17-R20.

Burke TR Jr, Li Z-H, Bolen JB and Marquez VE: Phosphonate-containing inhibitors of tyrosine-specific protein kinases, J Med Chem 1991;34:1577-1581.

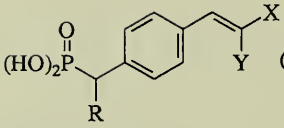
Burke TR Jr, Li Z-H, Bolen JB and Marquez VE. Structural Influences of styryl-based inhibitors on epidermal growth factor receptor and $p56^{lck}$ tyrosine-specific protein kinases, Bioorg Med Chem Lett 1991;1:165-168.

OPIATES



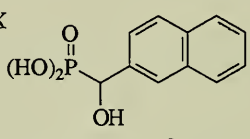
1

PHOSPHONATE-CONTAINING INHIBITORS



2

R = H, OH
X = CN, $CONH_2$
Y = CN, $CONH_2$



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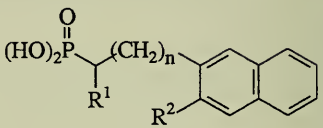
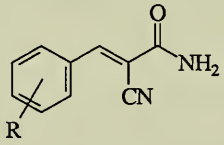


4

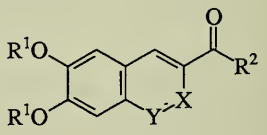
R^1; R^2 = H, OH
n = 0 - 2

POLYHYDROXYLATED AROMATIC INHIBITORS



5

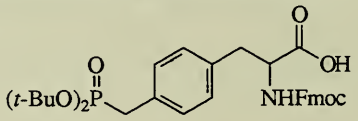
R = mono-, di-, trihydroxy



6

R^1 = H, Me
R^2 = OH, OMe, NH_2
X, Y = CH ; N

O-PHOSPHOTYROSINE MIMETIC



7

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 03581-22 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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
Other:	John S. Driscoll	Chief	LMC
	Harry Ford, Jr.	Senior Staff Fellow	LMC
	Jeri S. Roth	Chemist	LMC
	Pamela L. Russ	Chemist	LMC
	Fuming Xie	Visiting Fellow	LMC

COOPERATING UNITS *(if any)*
Toxicology Branch, DTP, DCT; Medicine Branch, COP, DCT; Pediatric Branch, COP, DCT; Preclinical Toxicology Division, Southern Research Institute

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.6	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 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 and clinical pharmacology, and methods enhancement), carbodine/isocarbodine (stereospecific synthesis and analytical characterization), and modified nucleosides (analytical characterization, purification of anomers and isomers, partition coefficient evaluation).

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. Cyclopentenyl Cytosine (CPE-C): Preclinical and Clinical Pharmacology and Enhancement of Current Analytical Methods:

a. CPE-C Continuous Infusion-Disposition and Pharmacokinetics in Dogs (Drs. Kelley, Tomaszewski, Page, Ms. Roth): The second stage of a toxicological and pharmacokinetics study on 24- and 72-hr continuous infusion CPE-C in beagle dogs confirmed the unusual plasma concentration *versus* time profile originally observed. CPE-C plasma concentrations did not increase smoothly to steady-state but showed a dip during the early part of the infusion indicating enhanced plasma elimination of drug, possibly from increased metabolism. No evidence of catabolism to cyclopentenyl uridine, the major metabolite in rhesus monkeys, was observed in dogs. Urinary excretion of unchanged CPE-C represented 8-35% of the administered dose and was lower than expected. This and the ability to detect low levels of CPE-C (> 0.1 μM) up to 48 hr after the end of infusion may indicate substantial sequestering of the drug, most likely as a phosphate, in a deep compartment.

b. Enhancement of Analytical Methods for Measuring CPE-C in Biological Fluids (Drs. Kelley, Xie): Research continues on the enhancement of CPE-C measurement in biological fluids, since the continuous infusion doses to be given to patients during Phase I clinical trials (see 1.c.) will probably result in drug plasma levels below the current limit of quantitation (0.1 μM). A new cytosine-nucleoside-specific naphthyletheno derivative has been evaluated both chemically and chromatographically. This derivative offers at least a 10-fold increase in sensitivity for CPE-C and cytidine when reverse phase HPLC and fluorescence detection are used. Biological sample preparation schemes compatible with this derivatization reaction are currently under investigation.

c. Proposed Disposition, Metabolism and Pharmacokinetic Study of Continuous Infusion CPE-C in an Adult Phase I Clinical Trial (Drs. Kelley, Xie and Grem): The protocol for a Phase I clinical trial of CPE-C is in place and will be activated upon FDA approval of the INDA. CPE-C will be administered to adult

cancer patients as a 24-hr continuous infusion every 3 weeks at a starting dose of 2 mg/m^2/hr.

d. Biochemical Studies with CPE-C (Drs. Ford, Cooney, Hao, Johns): The *in vitro* and *in vivo* toxicity of CPE-C can be modulated by pyrimidine nucleosides. For Molt-4 cells in culture, addition of 25 μM cytidine within 12 hr of CPE-C exposure is protective, while similar effects are observed in normal CDF_1 mice for bolus doses of cytidine administered 8 hr after CPE-C treatment (5 mg/kg, Daily X 9). Reverse phase HPLC methods have been developed for the measurement of cytidine, uridine and the corresponding deoxyribonucleosides in cell culture media, Molt-4 lymphoblasts and mouse plasma.

e. Stereospecific Synthesis of Carbodine and Isocarbodine (Drs. Kelley, Marquez, Ms. Russ): The selective catalytic addition of hydrogen to the double bond of the cyclopentenyl moiety of CPE-C has been investigated. Enantiomerically pure carbodine (hydrogenation of α-side) and isocarbodine (β-side reduction) have been isolated, analytically characterized, and evaluated for *in vitro* cytotoxicity. The order of cytotoxicity against L1210 cells in culture is CPE-C > carbodine > isocarbodine.

2. Automation of Laboratory Chromatography Capabilities Through Networked Personal Computers (Drs. Kelley, Musser, Ms. Roth): Low overhead networking architectures have been employed to interconnect laboratory PC's and to share peripheral devices. A more efficient multi-tasking software environment has been implemented for the LMC chromatography data system, and capabilities for graphics and pharmacokinetic analysis have been expanded. The mass spectrometer data system, based on a PDP 11/73 minicomputer, has been interfaced to laboratory PC's for mass spectral file transfer.

3. 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 and/or purified on an individual basis by the appropriate chromatographic techniques. Included in this group are modified nucleosides, neplanocin analogues, carboacyclic nucleosides, dideoxyribose nucleosides, and mono- and oligonucleotides.

Publications:

Blaney SM, Balis FM, Hegedus L, Heideman RL, McCully C, Murphy RF, Kelley JA, Poplack DG. Pharmacokinetics and metabolism of cyclopentenyl cytosine (CPE-C) in nonhuman primates, Cancer Res 1990;50:7915-7919.

Zaharko DS, Kelley JA, Tomaszewski JE, Hegedus L, Hartman NR. Cyclopentenyl cytosine: Interspecies predictions based on rodent plasma and renal kinetics, Inv New Drugs 1991;9:9-17.

Ward FT, Kelley JA, Roth JS, Lombardo FA, Weiss RB, Leyland-Jones B, Chun HG. A Phase I bioavailability and pharmacokinetic study of hexamethylene bisacetamide (NSC 95580) administered via nasogastric tube, Cancer Res 1991;51:1803-1810.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06177-06 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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
Other:	John S. Driscoll	Chief	LMC
	Harry Ford, Jr.	Senior Staff Fellow	LMC
	Jeri S. Roth	Chemist	LMC

COOPERATING UNITS *(if any)*
Clinical Oncology Program, DCT; Pediatric Branch, COP, DCT; Digestive Diseases Branch, NIDDK

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	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.)*

The objective of this project is the research and development of suitable analytical methods to: (1) establish the structure and purity of potential anti-AIDS agents and new antiviral drugs, (2) determine physical, 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 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. The role of adenosine deaminase in prodrug activation is under investigation.

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. Chemical and Physical Properties of Base-and Sugar-Modified Dideoxynucleosides (Drs. Kelley, Ford, Marquez, Ms. Roth): Characterization of the chemical and enzymatic stability of selected members of the dideoxynucleoside series incorporating a fluorine atom in the 2'-ß-position of the sugar continues. The acid stability (pH 1, 37°C) of several purine members of this series has been verified and relative rates of enzymatic deamination (cytidine deaminase) of selected cytidine analogues are being measured. Our rapid, microscale method for determining partition coefficients by HPLC has been further defined and extended. The general nature of this method has been demonstrated with non-nucleoside compounds. Partition coefficients (P) can also now be determined for the individual components of a mixture in a single micro-shake flask partitioning experiment. Isocratic HPLC mixture analysis, using a log P internal standard, results in improved precision and a means of directly comparing new compounds to standards measured by classical methods. Gradient HPLC mixture analysis permits the simultaneous measurement of P values, differing by as much as 100, and gives good agreement (± 0.1 log P unit) with values determined individually. Analytical and chemical evaluation of the ascorbic acid and sodium thiosulfate mixture used in an intra-venous Bulgarian AIDS treatment shows the ascorbic acid catalyzed production of 1% colloidal sulfur.

2. Analytical and Enzymatic Studies of 6-Substituted Dideoxypurine Nucleosides as Potential Lipophilic Prodrugs with Anti-HIV Activity (Drs. Kelley, Ford, Driscoll, Marquez, Mitsuya): The ability of a series of 6-substituted purine dideoxyurine nucleosides to inhibit the infectivity and cytopathic effect of HIV *in vitro* arises from their enzymatic conversion to ddI or ddG derivatives. The role of adenosine deaminase (ADA) in the activation of these prodrugs has been investigated by determination of the k_M, v_{max}, and relative rate of deamination for selected substrates, and by quantitation of this enzyme in the components of the *in vitro* HIV test system. Augmenting the *in vitro* test systems with additional ADA potentiates the anti-HIV activity of the prodrugs that are substrates for this enzyme.

Efforts are underway to correlate levels of ADA in these *in vitro* test systems with *in vivo* mammalian systems.

3. Disposition, Metabolism and Pharmacokinetics of 2′-F-ara-ddA in Non-human Primates (Drs. Kelley, Balis, Poplack, Ms. Roth): A study to investigate 2′-F-dd-ara-A disposition, metabolism and pharmacokinetics in male rhesus monkeys has been initiated. A reverse phase HPLC method employing C_{18} solid-phase extraction for sample isolation and concentration allows simultaneous measurement of 0.2 -40 μM 2′-F-dd-ara-A and its primary catabolite, 2′-F-dd-ara-I, in biological fluids. Both compounds could be measured in plasma, cerebrospinal fluid (CSF) and urine following treatment of an initial monkey with 20 mg/kg 2′-F-dd-ara-A administered as an i.v. bolus push. Plasma elimination of 2′-F-dd-ara-A is biexponential with a terminal half-life of 14 min, while that of 2′-F-dd-ara-I is monoexponential with a longer half-life of 40 min. While micromolar concentrations of 2′-F-dd-ara-I could be measured in CSF, the CSF:plasma ratio for either 2′-F-dd-araA or I was only 2-3%.

Publications:

Shirasaka T, Murakami K, Ford H Jr, Kelley JA, Yoshioka H, Kojima E, Aoki S, Broder S, Mitsuya H. Halogenated congeners of 2′,3′-dideoxy-purine nucleosides active against HIV in vitro: a new class of lipophilic prodrugs, Proc Natl Acad Sci USA 1990;87:9426-9430.

Murakami K, Shirasaka T, Yoshioka H, Kojima E, Aoki S, Ford H Jr, Driscoll JS, Kelley JA, Mitsuya H. Escherica coli-mediated biosynthesis and in vitro anti-HIV activity of lipophilic 6-halo-2′,3′-dideoxypurine nucleosides, J Med Chem 1991;34:1606-1612.

Barchi JJ Jr, Marquez VE, Driscoll JS, Ford H Jr, Mitsuya H, Shirasaka T, Aoki S, Kelley JA. Potential anti-AIDS Drugs. Lipophilic, adenosine deaminase-activated prodrugs, J Med Chem 1991;34:1647-1655.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06178-06 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Other: Pamela L. Russ	Chemist	LMC
Steven M. Musser	IRTA Fellow	LMC

COOPERATING UNITS *(if any)*

Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 confirmation and determination of nucleosides, nucleotides, natural products and synthetic intermediates and products. On-line micro-column liquid chromatography has been combined with a continuous-flow fast atom bombardment ionization interface for the analysis of nucleotides and conjugated metabolites. The use of quaternary amine surfactants to enhance sensitivity in fast atom bombardment mass spectrometry and increase separation and resolution in paired-ion reverse phase micro-column liquid chromatography has been evaluated.

Project Description:

General Objective:

The objective of this project is the development, evaluation and application of new mass spectral techniques for the determination of chemical structure, rapid analysis of complex mixtures, and measurement of trace components in biological systems. Fast atom bombardment mass spectrometry, alone and in combination with on-line micro-column liquid chromatography, is the technique of current interest.

Major Findings:

1. Continuous-Flow Fast Atom Bombardment Mass Spectrometry with On-line Liquid Chromatography for Mixture Analysis (Drs. Kelley, Musser): Micro-column liquid chromatography using both anion exchange and reverse phase techniques has been adapted for direct coupling to continuous flow fast atom bombardment mass spectrometry (FAB/MS). Mobile phases containing 1-5% glycerol and volatile buffers at flow rates of 3-5 µl/min are compatible with both the chromatography and the mass spectrometry. Initial studies have focused on the analysis of nucleotides since many anti-AIDS and antitumor agents are anabolized by phosphorylation and conjugation. The current limit of detection for these classes of compounds in static FAB is much too high for practical analysis of most material derived from *in vitro* or *in vivo* sources. Continuous flow FAB coupled with gradient anion exchange micro-column LC allows a 65X increase in full scan sensitivity for diphosphate conjugates under appropriate ionization conditions. Further FAB/MS sensitivity increases and enhancement of reverse phase micro-column LC are provided by quaternary amine surfactants. Subnanomolar sensitivity and on-line sample desalting is seen for mononucleotide analysis by combined micro-column LC and continuous flow FAB/MS. This technique has been employed to characterize the *in vitro* diphosphocholine and diphosphoethanolamine anabolites of 2′,3′-dideoxycytidine, a new anti-AIDS agent currently under clinical investigation.

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. To this end, negative ion FAB/MS techniques have been developed to analyze weakly acidic polyhydroxybenzilidene derivatives (tyrosine kinase inhibitors) with greatly increased sensitivity. Continuing studies on dehalogenation reactions of substituted nucleosides during FAB/MS analysis using negative ion spectra show radical anion formation indicating that dehalogenation is probably initiated by thermal electron capture.

Publication:

Fales HM, Sokoloski EA, Pannell LK, Klayman DL, Lin AJ, Brossi A, Kelley JA. Comparison of mass spectral techniques on organic peroxides related to artemisinin, Anal Chem 1990;62:2494-2501.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06194-03 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Modeling 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
Other:	Marc Nicklaus	Visiting Fellow	LMC
	John S. Driscoll	Chief	LMC
	Victor Marquez	Deputy Chief	LMC
	Terrence R. Burke	Research Chemist	LMC

COOPERATING UNITS *(if any)*

ITB, DTP, DCT, NCI

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Laboratory of Medicinal Chemistry

SECTION

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.85	1.85	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 capabilities of computer-assisted molecular modeling have been expanded in a variety of directions and the techniques have been applied to a number of problems in the LMC concerning drug design, receptor sites, binding to-and inhibition of enzymes.

Modeling Studies The effect of solvent on the stable conformations of various medicinally important molecules has been studied and some understanding of the conformation in solution has been secured.

Protein Kinase C Inhibition of the binding of phorbol to protein kinase C was observed with several ribose-derived γ-lactones as indicated by models of the compounds.

Tyrosine Kinase The structural requirements in the styrene series for successful inhibition of tyrosine kinase have been established and tested.

Reverse Transcriptase Inhibitors A study of the ring-expanded oxetanocins was completed. One of the synthesized isomers showed anti-HIV activity and the other did not. This result was compatible with the modeling data.

Three-Dimensional Database Approximately half the compounds in the DTP Database have been converted to three-dimensional structures and a distance-based search system has been developed.

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.

MAJOR FINDINGS

Modeling studies

The effect of solvent on the structure of small solute molecules has been studied by semi-empirical and *ab initio* methods. Polar solvents such as water can disrupt intramolecular hydrogen bonds and, insofar as these are important to the conformation of the solute molecule, may change the conformation. Otherwise, solvents appear to have relatively little effect upon the minimum energy structures of small molecules.

Design of inhibitors of protein kinase C

Numerous small molecules that possess the presumed pharmacophore for binding to protein kinase C in the site employed by phorbol have been modeled. Some of the better candidates for the role of inhibitor have been synthesized and tested and the results have provided support for the hypothesis that the crucial pharmacophore consists of two hydrogen bond acceptors and one donor with specific geometric dispositions. This information may now be used to identify other phorbol competitors.

Design of inhibitors of tyrosine kinase

An analysis of the structure-activity patterns shown by the styrene-related inhibitors of tyrosine kinase has led to an understanding of the effect of substituents upon the tyrosine kinase inhibitory activity of the compounds. This in turn, has provided a basis upon which new structures can be postulated.

Reverse transciptase inhibitors

Various analogs and homologs of the RT inhibitor, oxetanocin A have been modeled and the relationship of these compounds to both oxetanocin A and ddA has been established. Geometric requirements for anti-HIV activity have been developed and are being used in the design of putative RT inhibitors.

Three-dimensional database

Three-dimensional atomic coordinates for 125,000 "open" compounds in the DTP database have been developed and assembled into a "3D Database". The statistics of this conversion, the number of failures, the reasons for failure and the accuracy, vis-à-vis x-ray structures of about 2,000 of the coordinate sets have been documented. Completion of the 3D Database will be done by extramural DTP

(ITB) and a searchable version of the Database will then be installed on an NCI computer.

PUBLICATIONS

Heller SR, Milne GWA. Online searching on STN. A Beilstein reference manual, Springer-Verlag (New York) 1989. 289 pp. ISBN 3-540-50118-5.

Heller SR, Milne GWA. Online searching on Dialog. A Beilstein reference manual, Springer-Verlag (New York) 1990. 321 pp. ISBN 3-540-50122-3.

Tseng CK-H, Marquez VE, Milne GWA, Mitsuya H, Shirasaki T, Wysocki RJ, Driscoll JS. A ring-enlarged oxetanocin A analogue as an inhibitor of HIV infectivity, J Med Chem 1991;34:343-349.

Milne GWA. Very broad markush claims; A solution or a problem? Proceedings of a Round-Table Discussion Held on August 29, 1990, J Chem Inf & Comp Sci 1991;31: 9-30.

Milne GWA. Generic formulation of chemical composition, World Patent Info, in press

Héthelyi E, Milne GWA, Podányi B, Koczka I, Neszmélyi A. Composition of the essential oil of clone 409 of tanacetum vulgare. 2D NMR Investigation of trans-chrysanthenyl acetate, J Essential Oils, in press

Milne GWA, Driscoll JS, Marquez VE. Molecular modeling in drug design, Proceedings of the Montreux International Chemical Information Conference, Infonortics Ltd, Calne, England, 1989;19-40.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06195-03 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Polypeptides as Potential Anti-HIV and Antitumor Agents

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

Peter P. Roller		Research Chemist	LMC
Others:	George W. A. Milne	Research Chemist	LMC
	Motoyoshi Nomizu	Visiting Fellow	LMC

COOPERATING UNITS *(if any)*
AEB, DTP; LMVC, NCI; COP, DCT; LDBA, NIDR; ABL-FDRDC; Biology and Anomalies, NIDR

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	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.)*

A number of peptidic agents were designed, synthesized and tested in enzyme inhibition assays against the HIV-1 viral protease. 1. Design strategy utilized the generation of anti-sense peptides to six functionally significant regions of the enzyme. Of 66 peptides synthesized, 52 have been tested to date. The best inhibitors were found to be the hydropathically optimized, anti-sense peptides to the flap and the alpha helical region, and the peptides directed to bind to the polyprotein cleavage site (IC50 = 160-300 μM). Several all-D configuration peptides were effective as well. 2. Two cyclic substrate-based peptides and two of their linear analogs were synthesized to examine the effect of a constrained turn conformation at the enzymic cleavage site. 3. Ten short D- and L-configuration peptides, several of which contained gamma-glutamyl or beta-aspartyl building blocks at the cleavage site, were prepared as potential inhibitors.

Laminin is a large extracellular multidomain glycoprotein. Of the various active sites, the Ile-Lys-Val-Ala-Val (IKVAV) region in the A chain of laminin is associated with promoting cell attachment, migration, and collagenase IV induction. 1. We synthesized a heterotrimeric disulfide-linked 95-mer peptide encompassing the IKVAV region which also contained the highly alpha-helical 30-mer segments of both the B1 and B2 subunits. Competitive cell binding assays indicated enhanced receptor activity of the A chain when attached to the more structurally rigid B1-B2 chains. 2. Ten anti-sense peptides and several all-D configuration analogs of three receptor segments were prepared as potential inhibitors.

Project Description:

Objectives:

Design, synthesis and evaluation of effective inhibitors to HIV-1 protease. Evaluation of the "sense/anti-sense" concept of molecular recognition in the design of inhibitors. Synthesis of active site segments of laminin, and of inhibitors to laminin.

Major Findings:

Synthesis of HIV-1 protease inhibitors:
Using the "sense/anti-sense" concept of molecular recognition [Blalock and Smith (1984), and Fassina et al.(1989)], we designed and synthesized a large number of peptides that were specifically targeted to bind to six structural and/or functional domains of the enzyme. The targeted peptides were tested for enzyme inhibition using purified recombinant HIV protease. In addition, all-D configuration anti-sense peptides were evaluated for three regions.
a. Catalytic site region, and N- and C-terminal hinge region. Of 22 peptides synthesized and tested for enzyme inhibition, none showed appreciable inhibitory activity.
b. Flap region (res. 45-55). Of 16 peptides tested, the hydropathically optimized peptide, LDKASYSTNHL, was found to be the best inhibitor (IC50 = 160 uM). Its all-D configuration 11-mer analog was approx. 3 times less inhibitory. Modifications to this peptide diminished activity.
c. Alpha helical region (res. 85-94). Of 19 peptides synthesized, 6 have been tested. Significantly, peptides targeted to this region showed consistently good activities, with the best peptide being AYLSKEIASD (IC50 = 270 uM). Both the all-D configuration analog and the complementary RNA based peptide were only half as effective.
d. Gag p17/p27 cleavage site. Nine complementary 9-mer peptides were synthesized that were targeted to the cleavage site segment of the polyprotein itself. Preliminary results indicate that five of these are relatively good inhibitors (IC50 = 200-800 uM), and that the all D-configuration peptide is equally good when compared to the all L analog. Results to date indicate that the anti-sense approach of complementary peptide design can provide enzyme inhibitors with novel hitherto unknown types of structures. Inhibitors designed in this manner are weak relative to those obtained from substrate transition-state analog-based design. The hydropathic optimization method consistently gives better inhibitors compared to those based on complementary RNA. The all-D configuration analogs of effective peptides are also active, thus providing for proteolytically stable peptides.

Two cyclic and two linear p17/p24 substrate analog peptides were synthesized with the aim of testing if a constrained turn at the cleavage site affects the enzymic cleavage rate. Two of the peptides contained the transition-state analog amino acid, statine. The peptides are: Ac-Lys-Asn-Phe-Pro-Ile-Glu-amide (e), sequence as in e with Lys-1 ang Glu-6 side chains condensed (f), Ac-Lys-Asn-Sta-Pro-Ile-Glu-amide (g), sequence as in g with Lys-1 and Glu-6 side chains condensed (h).

Comparative enzyme inhibition assays have are not yet completed. However, these agents did not show in vitro inhibitory activities in HIV-1 infected ATH8 cells at 20-200 uM.

Ten short hydrophobic p17/p24 cleavage site segment analogs were prepared as potential protease inhibitors. Six of these were cleavage site dimers, Tyr-Pro, with variations of D- and L-amino acid configurations and with hydrophobic N- and C-terminal protective groups. This design was prompted by a recent report (Science, 249, 287, 1990), showing that certain Pro-Phe dimers interact with the viral coat protein, GP-120. Four tripeptides were synthesized which can be viewed as peptide backbone elongated analogs of cleavage site segments. These had the general structure, Z-Asn-D(or L)-gamma-Glu(or beta-Asp)-Pro-OBzl, with the Glu and Asp side chains O-benzylated. No enzyme inhibition assays have been done yet. All ten peptides were found to be inactive in virus-infected ATH8 cell culture assays.

Synthesis of potential inhibitors of laminin action:
Laminin is an extracellular protein with multiple functions. It has a number of receptor sites on its three disulfide-linked subunits (A, B1 and B2). In vitro it has been found to interact preferentially with tumor cells and to enhance the metastatic phenotype. Within subunit A there is a region encompassing Ile-Lys-Val-Ala-Val, that appears to promote cell adhesion, as well as increase experimental metastasis and collagenase IV activity. This segment is part of a region that forms an alpha-helical coiled-coil structure together with subunits B1 and B2. Biological studies are carried out collaboratively by Y.Yamada and coworkers (NIDR) on all prepared peptides. We synthesized a heterotrimeric disulfide linked 95-mer peptide comprised of amino acid residues 2081-2109 of the A chain and two 30-mer segments of the B1 and B2 chains. This trimer was demonstrated to have a stabilized alpha helical structure, using circular dichroism spectropolarimetry. Biological experiments confirmed the interpretation that "scaffolding" by the highly alpha helical B1-B2 chains increases the cellular receptor binding activity of the A chain. We prepared ten anti-sense peptides to the IKVAV region of laminin as potential inhibitors of tumor metastatic activity and all-D configuration analogs of three receptor segments (IKVAV, YIGSR and RGD) were prepared as potential proteolytically stable competitive inhibitors of laminin action.

Publications:

Keefer LK, Kroeger-Koepke MB, Ishizaki H, Michejda CJ, Saavedra JE, Hrabie JA, Yang CS and Roller PP. Stereoselectivity in the microsomal conversion of N-nitrosodimethylamine to formaldehyde, Chem Res Toxicol 1990;3:540-544.

Nomizu M, Inagaki Y, Yamashita T, Ohkubo A, Otaka A, Fujii N, Roller PP and Yajima H. Two-step hard acid deprotection/cleavage procedure for solid phase peptide synthesis, Int J Peptide Protein Res 1991;37:145-152.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06197-02 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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)*

N. Hartman	Sr. Staff Fellow	LMC
Other: David G. Johns	Medical Officer	LMC

COOPERATING UNITS *(if any)*
R. Yarchoan, H. Mitsuya, S. Kageyama, S. Broder, Clinical Oncology Program, NCI
C.-F. Perno, University of Rome, Rome, Italy, A. Fridland, St. Jude Children's Research Hospital, Memphis, TN

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	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.)*

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 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. The potential of interaction of ddI with ganciclovir and ranitidine was also investigated. It was determined that ddI had pharmacokinetic properties amenable to long term administration.

Project Description:

Objectives:

The objective of this study was the determination of human bioavailability of ddI in various oral formulations and the drug interactions of ddI with ranitidine and ganciclovir.

Major Findings:

2',3'-Dideoxyinosine (ddI) has shown activity against HIV disease in Phase I clinical trials. The drug is rapidly degraded by acid, however, thus raising questions as to the efficiency and reproducibility of its absorption after oral administration. This investigation studies the bioavailability of several oral dosage forms of ddI. When ddI was given to fasting patients as an oral solution with antacid, the bioavailability was 41±7% (mean±SEM). However, when given as buffered tablets the bioavailability was considerably less (25±5%). The bioavailability increased sightly when the tablets were given with supplemental antacid (36±6%). Two enteric coated preparations had reasonable bioavailability (36±5%, 26±5%), but the peak plasma levels were much lower and occurred at a much later time than with the oral solution. When ddI was given as a pre-measured powder containing sucrose and buffer to be reconstituted by the patient (the "sachet" preparation) the bioavailability was 29±6%. This was similar to that of the oral solution for this particular group of patients (30±7%). The bioavailability of the sachet was only 17±4% when administered with food, however. When the sachet was given to patients receiving ranitidine, no consistent change in bioavailability was noted. Also, no change in ddI pharmacokinetics was noted in patients receiving ganciclovir.

Publications:

Hartman NR, Yarchoan R, Pluda JM, Thomas RV, Wyvill KM, Flora KP, Broder S, Johns DG. Pharmacokinetics of 2',3'-dideoxyinosine in patients with severe human immunodeficiency infection. II. The effect of different oral formulations and the presence of other medications, Clinical Pharmacology and Therapeutics, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07181-06 LMC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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)*

D. G. Johns		Medical Officer	LMC
Other:	D. A. Cooney	Senior Investigator	LMC
	N. R. Hartman	Senior Staff Fellow	LMC
	Zhang Hao	Visiting Scientist	LMC
	H. Ford	Staff Fellow	LMC
	K. A. Dobyns	Chemist	LMC
	E. Gregory	Bio Lab Tech	LMC

COOPERATING UNITS *(if any)*
R. Yarchoan, H. Mitsuya, S. Kageyama, S. Broder, Clinical Oncology Program, NCI
C.-F. Perno, University of Rome, Rome, Italy, A. Fridland, St. Jude Children's Research Hospital, Memphis, TN, J. Plowman, PB, DTP

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	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.)*

1. We have explored the hitherto unexplained enhancement of the anti-HIV activity and the 5'-phosphorylation of 2',3'-dideoxypurine nucleosides by inosine monophosphate dehydrogenase (IMPD) inhibitors such as ribavirin, tiazofurin and mycophenolic acid. The latter agents, although themselves without anti-HIV activity, can stimulate the anti-HIV activity of ddI or ddG as much as 4-fold in appropriate assay systems. The enhancement appears to be due to an increased concentration of IMP arising as a consequence of IMPD inhibition by these agents, with IMP acting as the phosphate donor for the initial phosphorylation of ddI or ddG to ddIMP and ddGMP. Of the combinations examined, ddI/ribavirin may have the greatest potential for clinical use since both of these agents are in active clinical trial at the present time (ddI as an anti-HIV agent, and ribavirin in the treatment of other virus infections). 2. In view of the activity of 2',3'-dideoxynucleosides as anti-hepatitis B virus (HBV) agents, we have explored the activation of these agents in the isolated hepatocyte system. Unlike the relationship seen in the HIV system, the activity of these agents against HBV did not correlate with their efficiency of phosphorylation. ddG, for example, is phosphorylated relatively slowly, but is the most active of these agents against HBV. It appears likely that specificity is conferred by the dG termination of the negative strand of HBV, rendering this virus specifically sensitive to ddG analogues. 3. The pharmacology of cyclopentenyl cytosine (CPEC) (NSC 375575) has been explored extensively. The compound appears to act primarily via inhibition of CTP synthetase by its anabolite CPEC-TP. Cytidine effectively reverses the toxicity of the drug, even when administered several hours after CPEC. CPEC is scheduled for Phase I clinical trial in the fall of 1991.

1. Potentiation of the Phosphorylation and Antiviral Activity of Anti-HIV Agents by IMPD Inhibitors

As indicated in last year's report, the 5'-phosphorylation of the anti-HIV and anti-HBV agent 2',3'-dideoxyguanosine (ddG) is stimulated several-fold by inosine monophosphate dehydrogenase (IMPD) inhibitors such as ribavirin, tiazofurin and mycophenolic acid. This observation supports the proposal that the initial activation step for ddG (ddG → ddGMP) requires IMP as a phosphate donor, with the levels of the latter being greatly increased in the presence of IMPD inhibitors.

We have now extended these studies to 2',3'-dideoxyinosine (ddI), a compound which, unlike ddG, is in clinical trial at the present time as an anti-HIV agent. ddI, like ddG, requires IMP as a phosphate donor for its initial 5'-phosphorylation, a reaction catalyzed by a phosphotransferase (5'-nucleotidase) rather than by a purine nucleoside kinase. For ddI, as for ddG, the stimulation by IMPD inhibitors of the formation of the active metabolite was also increased (up to 4-fold) with a concomitant increase in anti-HIV activity. Of the combinations examined, ddI/ribavirin may have the greatest potential for clinical use since both of these compounds are in active clinical trial as single agents (ddI as an anti-HIV agent, and ribavirin as an agent useful in the treatment of other virus infections).

2. Activity of Antiretroviral Agents in Hepatocytes

2',3'-Dideoxynucleosides and their analogues (e.g. AZT) show activity in human hepatocyte assay systems in vitro and are also active in the duck hepatitis assay system both in vitro and in vivo (B. Lee et al., Antimicrob. Agents Chemother. 33: 336-339, 1989). In a recent NIDDK-sponsored clinical trial, ddI showed little activity in human hepatitis B; however in the hepatitis model systems studied to date, ddG and its analogs showed much greater activity than did ddI/ddA or the dideoxypyrimidine nucleosides (e.g. AZT, ddC). Thus the relative potency among this group of compounds against HBV differs greatly from the relative activity seen when they are tested against HIV. We have examined the antiviral activity of 2',3'-dideoxy-nucleosides in hepatocytes to determine whether metabolism accounts for this difference. In hepatocytes, phosphorylation of dideoxypyrimidine nucleosides was more efficient than that of dideoxypurine nucleosides, with formation of ddGTP from ddG being relatively poor. It would appear likely, therefore, in contrast to the situation with HIV, that the greater activity of ddG and its analogs in HBV model systems is due to termination by dG of the negative strand of HBV, and the specific interference by ddGTP with this process.

3. ddCDP-choline: ddCDP-ethanolamine

The anti-HIV agent ddC, presently in Phase II clinical trial, forms, in addition to 5'-phosphorylated anabolites, the two adducts ddCDP-choline and ddCDP-ethanolamine in T- and B-lymphocytes. Similar anabolic products are seen with the physiological parent compounds cytidine and 2'-deoxycytidine.

We have now achieved unequivocal identification of these two ddC adducts by isolation from large numbers of Molt-4 cells after treatment with ddC, followed by mass spectrometric comparison of the isolated metabolites with authentic synthetic ddCDP-choline and ddCDP-ethanolamine.

4. Studies with Cyclopentenyl Cytosine, NSC 375575

Cyclopentenyl cytosine, CPEC, is a synthetic nucleoside containing the base cytosine engrafted onto the unsaturated carbocyclic pseudosugar first found in the natural product neplanocin. CPEC is active in many animal tumor systems, and appears to act primarily via its 5'-triphosphate anabolite, CPEC-TP, an inhibitor of CTP synthetase, although a low level of incorporation of CPEC into cellular RNA is also detectable. Phase I clinical trials of CPEC are expected to begin in the summer of 1991. In the Molt-4 system, the compound demonstrates an IC_{50} ranging from 50 to 100 nM on 24 hr exposure to the drug. Cytidine, uridine and nitrobenzylthioinosine (a nucleoside transport inhibitor) prevent the cytotoxicity of CPEC when added before the drug, but only cytidine is an effective antidote when added at later times. CPEC-TP reaches relatively high concentrations within mammalian cells, as much as 50 times that of the parent drug in the medium and the half-life of CPEC-TP is relatively long (9-14 hr). A number of metabolites of CPEC and its deamination product, CPEU, are detectable, but appear to be without pharmacological activity.

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ANNUAL REPORT OF THE LABORATORY OF BIOLOGICAL CHEMISTRY

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

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 cellular processes that may contribute to the malignant properties of cancer cells. Specifically, the LBC focused on the biochemistry/biology of ADP-ribosylation factor (a small GTP-binding protein) and its role in protein secretion and metastasis; the stability of intercellular junctions and tumor invasion/metastasis; 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 two 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 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

approximately 500-fold and shown that the active enzyme is a 120 kDa dimer possibly made up of a mixture of 4-5 different charge and/or size isoenzymes. The enzyme has been shown to exist in tight association with a number of other proteins. This could have physiological significance with respect to the cytoplasmic translocation of N-myristoylproteins. We have found that a new compound, N-myristoyl tetrazole, inhibits HIV syncytia formation and protein N-myristoylation in tissue culture cells. While the tetrazole does not inhibit NMT, it does inhibit fatty acyl coenzyme A synthetase suggesting that the N-myristoylation dependent phenomenon might be indirectly blocked in vivo by inhibition of the formation of the NMT co-substrate, myristoyl CoA. Several additional fatty acid inhibitors of fatty acyl CoA synthetase also have been characterized in vitro. The HIV p17gag and p27nef N-myristoylated proteins have been expressed 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 subcellular membrane "acceptors". Histochemical analysis of COS cells transfected with p27nef vectors showed p27nef to be localized to the perinuclear membranes. In contrast, a gly^2 to ala^2 mutant of p27nef is localized to the nucleus. The DNA-binding of a new 32 Kda transcriptional factor to HIV-LTR is markedly enhanced in CEM clones stably transfected with p27nef but not in cells stably transfected with the $p27nef^{ala^2}$ mutant. This factor may play a role in the down-regulation of HIV replication by p27nef.

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 are at least 20 retinoylated proteins which are primarily localized in the nucleus. Based on chemical property, the RA moiety is probably linked to protein *via* a thioester bond. We have now found that some of these retinoylated proteins are cAMP-binding proteins. Five proteins, covalently labeled by 8-azido-[^{32}P]cAMP which specifically reacts with the regulatory subunits of cAMP-dependent protein kinase, comigrated on two-dimensional polyacrylamide gel electrophoresis with retinoylated proteins of Mr 37,000 ($p37^{RA}$), 47,000 ($p47^{RA}$), and 51,000 ($p51^{RA}$) labeled by ^{3}H-retinoic acid treatment of intact cells. Furthermore, $p47^{RA}$ coeluted on Mono Q anion exchange chromatography with the type I cAMP-dependent protein kinase holoenzyme and $p51^{RA}$ coeluted on Mono Q anion exchange chromatography with the type II cAMP-dependent protein kinase holoenzyme. An antiserum specific to RI, the cAMP-binding regulatory subunit of type I cAMP-dependent protein kinase, immunoprecipitated $p47^{RA}$. An antiserum specific to RII, the cAMP-binding regulatory subunit of type II cAMP-dependent protein kinase, immunoprecipitated $p51^{RA}$.

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 recently documented important functional distinctions between families that should facilitate further studies but we continue to focus our efforts on one sub-family of the monomeric 20-25 Kda proteins, the ARF family. ARF is the only one of the more than fifty known smaller GTP binding proteins with a defined biochemical function. ARF is a cofactor in the covalent modification of the human proto-oncogene, *gsp*. In addition, we have recently proven that ARF can be directly activated by the anti-metastatic gene, *nm23*. We have discovered that in humans this family consists not only of the three previously identified gene products, but of more than 15 structurally related genes and their products. Thus, we are engaged in cloning and sequencing this newly enlarged family to begin to document its size, functions and importance to cell physiology and pathophysiology.The ARF proteins are a sub-family of structurally and functionally conserved 21 Kda GTP binding regulatory proteins. ARF has now been shown to have essential functions in the maintenance of the integrity and proper functioning of the Golgi complex. Evidence supporting this has come from immunocytochemistry, in which ARF has been localized to the cytosolic surface of cis-Golgi cisternae and is the most abundant coat protein on Golgi-derived vesicles; genetics, in which mutant ARFs have been shown to cause errors in processing of secreted proteins; and more recently biochemistry, in which an in vitro assay for vesicle mediated secretion and fusion has been utilized to demonstrate both an essential role for ARF proteins as well as its putative site of regulation.

Intercellular junctions play an important role in the function, stability and integrity of epithelial tissues. Invasive behavior can be produced in cultured epithelia by disrupting intercellular junctions. An understanding of the mechanism(s) involved in stabilizing intercellular junctions may lead to the rational design of drugs with anti-invasive and anti-metastatic activities. This past year, we developed criteria for assessing junctional stability in cultured epithelia, developed a quantitative assay that reflects junctional stability, and investigated whether particular junctions could be selectively stabilized in a Ca++-depleted environment. The significant results of this study are: (1) the demonstration that desmosomal junctions can be selectively stabilized by at least two different mechanisms; the first, a rapid biophysical response mechanism mediated by organic anions, that prevents desmosome splitting and internalization and the second, a slow metabolic response dependent upon inhibition of mevalonate metabolism, which maintains the original positions of half-desmosomes on cell surfaces and allows the rapid reformation of junctions when effective Ca++ levels are restored; and (2) the discovery that desmosomal maturation may be an integral part of epithelial tissue differentiation insofar as it involves the acquisition of mechanisms for controlling the stability of desmosomal junctions independently of other types of intercellular junctions.

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. Enzymes of the pathway responsible for hyaluronate synthesis are under study to determine their response to mitogen-stimulation and as targets for drug design. Analogues of nucleoside diphosphate sugars have been prepared and are under evaluation for their ability to inhibit glycosaminoglycan synthesis. Active agents will be evaluated for anti-invasive and anti-metastatic effectiveness.

The relative contribution of de novo synthesis to tissue pyrimidine nucleotide pools is an important parameter in the rational design of anti-pyrimidine therapies. Both pathways were measured in tumored and non-tumored mice using stable isotopes to quantitate de novo synthesis and radioisotopes to quantitate salvage. All murine tumors studied rely predominantly on de novo synthesis and most do not switch to the salvage pathway when the de novo pathway is blocked. One murine tumor (Nettesheim carcinoma) did respond to PALA treatment by stimulating salvage; this tumor will be used to determine the therapeutic benefit of concurrent inhibition of both pathways. The normal mouse tissues studied utilize a balance of both pathways and do not enhance salvage in response to a block of de novo. Dietary protein was found to enhance de novo synthesis in intestine and to protect mice from the toxic effects of PALA and 5-FU; there was no enhancement in B-16 melanoma. Thus dietary protein could be used to enhance the differential toxicity of these agents.

The preceding summary outlines the objectives of the Laboratory of Biological Chemistry and describes some of the research carried out within the Laboratory during the past year. The individual Project Reports, which follow, describe this research in greater detail.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06163-07 LBC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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:	M. August	Sr. Staff Fellow	LBC NCI
	C. Chisena	Biologist	LBC NCI
	K. Duncan	IRTA Fellow	LBC NCI
	M. Grubb	IRTA Fellow	LBC NCI
	J. Kitchen	IRTA Fellow	LBC NCI
	N. Malinowski	Chemist	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:
7.5	7.5	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.)*

This project is composed of two major areas of research: (1) studies of pyrimidine nucleotide synthesis in vivo and (2) cellular control of nucleoside diphosphate sugar synthesis and glycosaminoglycan formation.

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. Both pathways were measured in the intact mouse using stable isotopes to quantitate de novo activity and radiolabled uridine to quantitate salvage. All murine tumors studied to date rely on de novo synthesis and most do not switch to the salvage pathway when the de novo pathway is blocked. One murine tumor (Nettesheim carcinoma) responded to PALA treatment by stimulating salvage; this tumor will be used to determine the therapeutic benefit of concurrent inhibition of both pathways. Normal mouse tissues studied utilize a balance of both pathways and do not enhance salvage in response to a block of de novo. Dietary nitrogen was found to enhance de novo synthesis in intestine and to protect mice from the toxic effects of PALA and 5-FU; there was no enhancement in B-16 melanoma. Thus dietary nitrogen could be used to enhance the differential toxicity of these agents

Data from our Laboratory support a link between mitogen-stimulated synthesis of the glycosaminoglycan hyaluronate and the activation of pathways responsible for the synthesis of nucleoside diphosphate sugars which are precursors for hyaluronate and the oligosaccharide moieties of other GAGs. Data indicate that hyaluronate synthesis in fibroblasts can be stimulated by regulatory peptides released by carcinomas. Potential inhibitors of enzymes of the hyaluronate pathway are under study for their ability to block mitogen-induced GAG synthesis. Active agents will be evaluated for anti-invasive and anti-metastatic properties.

Objectives:

This Project is composed of two major areas of research:

I. Studies of pyrimidine nucleotide synthesis in vivo. The overall objective of this area of research 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. Research 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.

II. Cellular control of nucleoside diphosphate sugar synthesis and glycosaminoglycan formation. The overall objective of this area of research is to understand the regulation of glycosaminoglycan synthesis (particularly hyaluronate) in fibroblasts and to determine if modulation of glycosaminoglycan synthesis can alter the invasive and metastatic properties of cancer cells. Research is divided into the following specific aims: to study the tumor-fibroblast interaction and determine which factors/combinations of factors secreted by tumor cells stimulate GAG synthesis in fibroblasts; to study the regulation of pathways that provide precursors for GAG synthesis; to design, identify, and evaluate agents to alter hyaluronate synthesis and determine if they alter the invasive and metastatic properties of cancer cells in vivo.

Major Findings:

I. Studies of pyrimidine nucleotide synthesis in vivo.

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. The contribution of each pathway can be determined in the intact animal by using L-[15N]alanine to quantitate de novo pathway activity and 14C-uridine to quantitate salvage activity (see 1990 Annual Report). Using this approach we found that murine tumors (L1210, B16, P388, Nettesheim) utilize de novo synthesis as the predominant pathway (4 to 5-fold de novo vs salvage) whereas normal mouse tissues utilize a balance of both pathways, usually in favor of salvage. When this measurement is repeated 24 hours after a 400 mg/kg dose of N-phosphonacetyl-L-aspartic acid (PALA), de novo synthesis is substantially inhibited in all tissues and tumors after this treatment, although significant residual activity is observed in intestine and L1210 cells. Remarkably, the Nettesheim carcinoma was the only tumor or tissue to show a significant increase in salvage synthesis after PALA

treatment. Thus, stimulation of salvage synthesis is not the usual response to inhibition of de novo synthesis as would be predicted. From these and other data collected to date, it appears that the extent and duration of inhibition of pathway activity is related to antitumor activity. Thus, enzymes of the de novo pathway are viable targets for antitumor drug design and inhibitors that exploit differences between tumor and host tissues could be useful antitumor agents.

For those tumors (e.g. Nettesheim) that respond to PALA by increasing salvage, concurrent inhibition of both pathways could be of therapeutic value. After a lengthy search for inhibitors of uridine kinase, we found one such inhibitor (CPE-U) with practical utility in vivo. The combination of a non-toxic dose of PALA with a non-toxic dose of CPE-U resulted in animal deaths within 3 to 4 days of the start of a continuous exposure to both agents. Future experiments will determine if concurrent inhibition of both pathways is of therapeutic benefit.

We found previously (see 1990 Annual Report) that increasing dietary nitrogen protected mice from 5-FU and PALA toxicity due to CPS-I activity in the intestine. Studies during the past year show that increasing dietary protein increases de novo pyrimidine synthesis in liver and intestine but not in transplanted murine tumors. Likewise, inhibition of de novo synthesis by PALA is reduced by high dietary protein in intestine but not in tumors. Thus, dietary nitrogen can be used to enhance the differential toxicity of 5-FU or PALA.

II. Cellular control of nucleoside diphosphate sugar synthesis and glycosaminoglycan formation.

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.

Serum, conditioned medium from carcinoma cells, growth factors, and combinations of growth factors were evaluated for their effect on UDP-glucuronic acid synthesis and hyaluronate production by fibroblasts. The data indicate a link between the UDP-glucuronic acid synthesis and hyaluronate synthesis. Certain combinations of growth factors were synergistic in their effect. It appears that hyaluronate synthesis is determined by combinations of growth factors present in serum and that are released by carcinomas. Also, the response varies with the type of fibroblast.

Assays for enzymes leading to the synthesis of UDP-sugars and hyaluronate were developed to further investigate the mechanism responsible for the stimulation of UDP-sugar and hyaluronate synthesis. An evaluation of UDP-glucose dehydrogenase was completed. The other enzymes of the pathway are under study. These assays will also be used to evaluate agents designed as enzyme inhibitors.

In a search for inhibitors of hyaluronate synthesis, a series of UDP-sugar analogues were synthesized which should be stable in serum and have increased membrane penetration. These agents are currently under evaluation for biological activity (enzyme inhibition, effects on GAG synthesis) and for serum stability and cellular transport. Vanadate (a reported stimulator of GAG synthesis) was found to be an inhibitor of hyaluronate synthesis when the specific activity of the precursor (UDP-glucuronic acid) was determined and the hyaluronate quantitated directly. Thus, it is important to take into account precursor specific activity and culture conditions when evaluating the effect of an agent on GAG synthesis.

Publications:

Zaharevitz, D.W., Anderson, L.W., Strong, J.M., Hyman, R. and Cysyk, R.L. De novo synthesis of uracil nucleotides in mouse liver and intestine studied with the use of ^{15}N-labelled amino acids, Eur J Biochem 1990;189:437-440.

Zaharevitz, D.W., Chisena, C.A. and Cysyk, R.L. Rapid increase of cellular UDP-glucuronide after mitogen stimulation of quiescent 3T3 mouse fibroblasts, Biochem Int 1990;20:1067-1076.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06167-07 LBC

PERIOD COVERED

October 1, 1990 to September 30. 1991

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

Myristoylation-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 Fellow	LBC, NCI
	G. Yu	Visiting Associate	LBC, NCI
	S. Bouziane	Visiting Fellow	LBC, NCI
	S. Sturch	Microbiologist	LBC, NCI

COOPERATING UNITS *(if any)*

Program Development Research Group, DTP, NCI

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	6	.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.)*

The co-translational amide linkage of myristic acid to an N-terminal glycine certain tyrosine kinase onc- and retroviral gag-proteins (i.e. N-myristoylation) has been shown to play an essential role in the targeting of cytoplasmic onc-kinases and retroviral gag structural proteins to various subcellular membrane compartments. Site directed mutagenesis designed to b lock N-myristoylation prevents membrane binding and inhibits both cellular transformation and viral replication. We have purified N-myristoyl-transferase (NMT) from cow brain approximately 500-fold and shown that the active enzyme is a 120 kDa dimer possibly made up of a mixture of 4-5 different charge and/or size isoenzymes. The enzyme has been shown to exist in tight association with a number of other proteins. This could have physiological significance with respect to the cytoplasmic translocation of N-myristoylproteins. We have found that a new compound, N-myristoyl tetrazole, inhibits HIV syncytia formation and protein N-myristoylation in tissue culture cells. While the tetrazole does not inhibit NMT, it does inhibit fatty acyl coenzyme A synthetase suggesting that the N-myristoylation dependent phenomenon might be indirectly blocked in vivo by inhibition of the formation of the NMT co-substrate, myristoyl CoA. Several additional fatty acid inhibitors of fatty acyl CoA synthetase also have been characterized in vitro. The HIV p17gag and p27nef N-myristoylated proteins have been expressed 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 subcellular membrane "acceptors". Histochemical analysis of COS cells transfected with p27nef vectors showed p27nef to be localized to the perinuclear membranes. In contrast, a gly^2 to ala^2 mutant of p27nef is localized to the nucleus. The DNA-binding of a new 32 kDa transcriptional factor to HIV-LTR is markedly enhanced in CEM clones stably transfected with p27nef but not in cells stably transfected with the $p27nef^{ala2}$ mutant.

Objectives.

In this project we will examine the role of N-myristoylation on onc-tyrosine kinases and retroviral gag structural proteins as a target for the chemotherapeutic inhibition of cellular transformation and viral reproduction. Specific goals include (i) studying the biochemistry and molecular genetics of N-myristoyl transferase (NMT); (ii) developing inhibitors to myristoyl CoA synthetase and NMT; (iii) characterizing the mechanism of cytoplasmic translocation of N-myristoylproteins; (iv) characterizing the mechanism(s) of N-myristoylprotein membrane binding, and (v) identifying the functional mechanisms of N-myristoylated onc- and gag-proteins.

Major Findings.

1. 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 myrisotyl analog, 1-bromo-2-pentadecanone, and the 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 a 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 non-competitive inhibitor, 1-bromo-2-pentadecanone, also inhibited NMT in an irreversible time dependent manor with a k_{+1} = 2 x 10-4 min-1.

2. The complete coding region of $p17^{gag}$ was cloned into the pET3c expression vector and was used to overexpress HIV-1 $p17^{gag}$ in E. coli. Induction of the PET3C vector in transfected bacteria caused the accumulation of a 17 kDa polypeptide in the soluble cell fraction which was released by sonication in hypotonic non-detergent buffer. The 17 kDa polypeptide was purified by ammonium sulfate precipitation between 55% to 85% saturation and successive chromatography on G-75 Sephadex, DEAE Sephacel, and S-Sephadex. The final product was purified 12-fold with about a 25% recovery from the original soluble cell lysate and was judged to be 97+% pure by SDS-PAGE. Western blotting with two different antibodies confirmed the identity of the purified 17 kDa polypeptide as authentic $p17^{gag}$. The bacterially expressed $p17^{gag}$ was quantitatively N-myristoylated *in vitro* in the presence of myristoyl CoA and mammalian N-myristoyltransferase with a pseudo-first order rate constant of $4.66 \pm 1.0 \times 10^{-3}$ min^{-1}. The N-terminal myristoylglycine linkage was confirmed by identification of the p-nitrobenzylazlactone derivative of N-myristoylglycine in the partial acid hydrolysate of the purified *in vitro* N-myristoylated $p17^{gag}$.

Proposed Course.

N-Myristoylation plays a role in a number of important cellular control phenomenon. The fact that a fatty acid that makes up less than 3 % of the total cellular fatty acid pool is utilized with such absolute specificity by transforming onc-kinases suggest it has a central role in the control of cellular growth and differentiation. Furthermore the involvement of N-myristoylation in mammalian retrovirus replication makes this phenomenon attractive as a target for drug development directed at a number of potential diseases. The work on this project is designed to clarify several specific aspects concerning the process of N-myristoylation. From these studies, it may be possible to design novel new compounds for specifically blocking this lipid modification. These agents may then provide us with a pharmacologic means to specifically treat N-myristoylation dependent cellular transformation and/or mammalian retroviral related disorders.

We propose to investigate four specific key aspects of the N-myristoylation pathway which may be susceptible to direct chemotherapeutic manipulation. These include, (i) inhibition of the myristoyl CoA synthetase(s), (ii) inhibition of NMT, (iii) inhibition of cytoplasmic translocation of N-myristoylproteins, (iv) inhibition of the binding of N-myristoylproteins to specific membrane "acceptors", and (v) identifying the biological activities of N-myristoylated proteins. We would predict that these properties are essential to the overall N-myristoylation pathway and therefore might represent opportunities to block the resulting lipoprotein dependent transformation and/or viral replication.

(i) Myristoyl CoA Synthetase. An assay for myristoyl CoA synthetase will used to test for possible enzyme inhibitors. Specifically identified inhibitors will be tested for their effect on protein N-myristoylation and HIV replication in tissue culture.

(ii) N-Myristoyl Transferase(s). An assay for NMT will be used to test for specific NMT inhibitors. The mammalian NMT will be further purified and characterized with respect to its physical, chemical and kinetic properties and compared with the previously characterized yeast enzyme. Specific antibodies and photoactive peptide probes will be used to characterize for the presence of NMT size and charge isozymes.

(iii) N-Myristoylprotein Cytoplasmic Translocation. N-Myristoylprotein associations other cytoplasmic proteins will be studied with specific antibodies in order to evaluate the role of soluble NMT-complexes in the translocation of the N-myristoylproteins from their site of synthesis in the cytoplasm to their specific subcellular membrane targets.

(iv) N-Myristoylprotein Membrane Binding. Membrane binding sites for the HIV p17gag and p27nef and the Rous sarcoma virus p60src N-myristoylproteins will be studied using bacterial expressed proteins and specific synthetic peptides as ligand probes. Mammalian vectors containing different HIV gag deletion

mutants will be used to characterize the subcellular distribution of mutant gag products. N-Myristoylprotein membrane "acceptors" will be identified, purified, characterized, and cloned. Specific agents will be designed to block N-myristoylprotein "acceptor" interaction. These will be tested as inhibitors of HIV replication in tissue cultured cells.

(v) N-Myristoylprotein Biological Functions. The effect of N-myristoylation on the role of nef in the regulation of HIV replication will be examined at the transcriptional level. The apparent subcellular targeting of N-myristoylated and non-myristoylated p17gag to the nucleus will be characterized. The binding of N-myristoylated proteins with membrane "acceptors will be characterized with respect to changes in protein phosphorylation.

Publications.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06181-06 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	Jenny Clark	Microbiologist	LBC, NCI
	Margaret Cavenagh	Biologist	LBC, NCI
	Paul Randazzo	Sr. Staff Fellow	LBC, NCI
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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

This section is involved in studies of the functions and mechanisms of action of members of the superfamily of regulatory GTP binding proteins. We have recently documented important functional distinctions between families that should facilitate further studies but we continue to focus our efforts on one sub-family of the monomeric 20-25 kDa proteins, the ARF family. ARF is the only one of the more than fifty known smaller GTP binding proteins with a defined biochemical function. ARF is a cofactor in the covalent modification of the human proto-oncogene, *gsp*. In addition, we have recently proven that ARF can be directly activated by the anti-metastatic gene, *nm23*. We have discovered that in humans this family consists not only of the three previously identified gene products, but of more than 15 structurally related genes and their products. Thus, we are engaged in cloning and sequencing this newly enlarged family to begin to document its size, functions and importance to cell physiology and pathophysiology.The ARF proteins are a sub-family of structurally and functionally conserved 21 kDa GTP binding regulatory proteins. ARF has now been shown to have essential functions in the maintenance of the integrity and proper functioning of the Golgi complex. Evidence supporting this has come from immunocytochemistry, in which ARF has been localized to the cytosolic surface of cis-Golgi cisternae and is the most abundant coat protein on Golgi-derived vesicle; genetics, in which mutant ARFs have been shown to cause errors in processing of secreted proteins; and more recently biochemistry, in which an in vitro assay for vesicle mediated secretion and fusion has been utilized to demonstrated both an essential role for ARF proteins as well its putative site of regulation. Further elaboration of the number and functions of ARF proteins and any mediators of ARF action include further characterization of the three suppressors of ARF mutations in yeast which were discovered in this lab, purification of an ARF GAP (GTPase activating protein) activity, first documented in this lab, and a search for any activators of ARF, in addition to the NDK proteins.

Introduction

The presence of GTP binding proteins as control points in a myriad of cellular processes or pathways is rapidly becoming the rule. These regulatory GTP binding proteins appear to control such processes as the protein secretory pathway, cellular proliferation, tumor metastasis, and signal transduction at the cell surface involving more than 100 different agonist which modulate secondary messenger systems including adenylate cyclase, phospholipases, and a number of ion channels. The regulatory GTP binding proteins can be divided into two structurally and functionally distinct families; the G proteins, the trimeric signal transducers which include Gs, Gi, Go, Gq, Gt and others with α subunits encoded by at least 16 distinct but homologous cDNAs, and the monomeric 20-25 kDa GTP binding proteins, with cDNAs which encode more than 50 distinct proteins. This latter family can be further divided, based on structural considerations, into four sub-families; the RAS and related proteins, the RAB and related proteins, the RHO and related proteins, and the ARF and related proteins.

As the size of this family of regulatory proteins grows and the similarity in sequences becomes even more evident, it is increasingly important to define functional properties of these proteins so that we may understand the need and role of such a redundant structural theme. The ARF proteins are the only members of the 20-25 kDa monomeric GTP binding protein family with a defined biochemical function. That is, cofactor in the ADP-ribosylation of Gs by cholera toxin. As Gs has recently been shown to be the proto-oncogenic form of the *gsp* oncogene, this is the first indication of a direct interaction of ARF with a human oncogene. ARFs are ubiquitous in eukaryotic cells and serve an essential function in both species where such tests are readily performed, *Saccharomyces cerevisiae* and *Drosophila melanogaster*.

Attempts to identify changes in the levels of specific mRNAs which correlate well with metastatic potential have led to the description of the *nm23* gene as an anti-metastatic gene in the mouse. The level of its message was found to be inversely correlated with the metastatic potential and is thus called an anti-metastatic gene. Sequencing of the *nm23* coding region revealed a close structural relationship to human nucleoside diphosphate kinases (NDKs). Dr. Patricia Steeg, NCI, who first identified *nm23*, as well as others are engaged in studies aimed at elucidating the mechanism of action of this gene as an anti-metastatic gene. Results in Dr. Lance Liotta's have previously suggested a role for a GTP binding protein in the metastatic processes. As we have recent evidence proving that NDKs can directly activate the ARF proteins, we have initiated a collaboration with Dr. Steeg's section to more fully explore the relationship between ARF and the human NDK genes and their products.

Objectives

The objectives of this section of LBC are to utilize a multi-disciplinary approach, including techniques of molecular biology, cell biology, genetics, and biochemistry to describe the ARF family of proteins, understand their site and mechanisms of action, and define their roles in cell physiology and pathophysiology. The multi-discipline approach is designed both to allow a

clearer picture of ARF function and also to allow us to rapidly expand our studies into new areas as new leads are discovered.

Major Findings

1) **ARF proteins were shown to be activated by direct phosphorylation of bound GDP by nucleoside diphosphate kinases, including the anti-metastatic gene product of the mouse *nm23* gene.** Thus, a novel mechanism of activation of a regulatory GTP binding protein has been demonstrated and ARF has been implicated as a potential mediator of the action of the anti-metastatic properties of NDK proteins.

2) **The mouse *nm23* gene and two human homologues, termed hNDK1 and hNDK2, were over-expressed in bacteria, purified in milligram amounts to near homogeneity, and demonstrated for the first time to have nucleoside diphosphate kinase activity.** These recombinant proteins have been used both in our lab to study the mechanism of action of NDK as an activator of ARF and in Dr. Patricia Steeg's laboratory to further studies on the role of NDK genes in metastasis.

3) **The polymerase chain reaction has been used to clone fragments of twelve new ARF-related cDNAs in human and two more in yeast.** These fragments are now being used to clone full length cDNA or genomic clones for these genes. Thus, it is clear that the ARF family is much larger than previously thought. A full description of the family is necessary if we are to fully understand the roles of ARFs in the cell and possible roles in pathophysiology, e.g. metastasis. It is likely that ARF-related gene products will play important roles in cellular processes not yet identified.

4) **The functional and structural conservation of ARF proteins have been demonstrated between yeast and man.** The first stringent functional definition of a sub-family of smaller GTP binding proteins was described. Such functional information is critical in the unraveling of the large number of smaller GTP binding proteins and the ultimate description of their cellular roles.

5) **At least three new genes have been cloned and sequenced in yeast as suppressors of an temperature sensitive ARF1 point mutation.** Mutations in these genes are currently being constructed both to examine the function of these genes in yeast as well as to test their role in ARF regulated functions, e.g. protein secretion. Construction of better temperature sensitive alleles of ARF1 is underway to allow further screening for genes which encode components of the ARF pathway.

6) **ARF has been shown to be a critical regulator of vesicle budding and fusion in *in vitro* assays of protein secretion or vesicle fusion.** A peptide was synthesized from an important domain of ARF and shown to have anti-ARF activity in a number of assays. This peptide, along with recombinant ARF and myristoylated ARF proteins, have been used to document specific roles for ARF proteins in vesicle budding and fusion. The structure of this peptide has also been studied as a prelude to the design of specific ARF antagonist.

7) **ARF was shown to be the most abundant of the coat proteins on Golgi-derived coated vesicles.** Binding of ARF to both Golgi cisternae and coated vesicles was further shown to be sensitive to Brefeldin A, a potent, reversible inhibitor of protein secretion.

Future plans

1) **Investigate the functional relationship between ARF and products of the mouse *nm23* and human NDK genes.** This involves both further biochemical characterization of the interactions between these proteins and consequences of the activation of ARF by NDKs, and also looking for correlations between the level of human NDKs expressed in tumor cells and the number, level or degree of activation of the ARF proteins present.

2) **Further characterize the ARF family of regulatory GTP binding proteins.** This will include the continued cloning and sequencing of the fourteen newly identified genes, expression of these new genes in bacteria and yeast to allow functional studies, and the generation of specific antibodies to allow immunocytochemical and immunohistochemical screening.

3) **Identification and characterization of new genes or proteins which are involved in the ARF pathway in eukaryotes.** This will include both biochemical and genetic techniques to identify specific genes or proteins, respectively. Specifically, we are concentrating on the role of the three suppressors of ARF mutations that we have already cloned and purification of the mammalian ARF GAP protein(s).

Publications

1. Kahn, R.A. The ADP-ribosylation factor of adenylate cyclase: a 21 kDa GTP-binding protein. In: Birnbaumer, L. and Iyengar, R. (Eds.) G Proteins. San Diego, CA:Academic Press, 1990;201-214.

2. Kahn, R.A. Purification and quantitation of the ADP-ribosylation factor of adenylate cyclase. Methods in Enzymol., in press.

3. Stearns, T., Willingham, M.C., Botstein, D., and Kahn, R.A. The ADP-ribosylation Factor (ARF) is Physically and Functionally Associated with the Golgi Complex, Proc Natl Acad Sci (USA) 1990;87:1238-1242.

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6. Knight, M., Gluch, S., Takahashi, K., Dang, T.T., and Kahn, R.A., Purification of a synthetic myristoylated peptide by countercurrent chromatography, J Chromatography 1991;538:141-147.

7. Tamkun, J.W., Kahn, R.A., Kissinger, M., Brizuela, B.J., Rulka, C., Scott, M.P., and Kennison, J.A., Proc Natl Acad Sci (USA) 1991;88:3120-3124.

8. Regazzi, R., Ullrich, S., Kahn, R.A., and Wollheim, C.B. Redistribution of ADP-ribosylation factor during stimulation of permeabilized cells with GTP analogues. Biochemical J 1991, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 07156-08 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 Associate	LBC, NCI
	He Ruyi	Visiting Fellow	LBC, NCI
	Donald Stump	IRTA Fellow	LBC, NCI

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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

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.)*

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 are at least 20 retinoylated proteins which are primarily localized in the nucleus. Based on chemical property, the RA moiety is probably linked to protein *via* a thioester bond. We have now found that some of these retinoylated proteins are cAMP-binding proteins. Five proteins, covalently labeled by 8-azido-[^{32}P]cAMP which specifically reacts with the regulatory subunits of cAMP-dependent protein kinase, comigrated on two-dimensional polyacrylamide gel electrophoresis with retinoylated proteins of Mr 37,000 ($p37^{RA}$), 47,000 ($p47^{RA}$), and 51,000 ($p51^{RA}$) labeled by ^{3}H-retinoic acid treatment of intact cells. Furthermore, $p47^{RA}$ coeluted on Mono Q anion exchange chromatography with the type I cAMP-dependent protein kinase holoenzyme and $p51^{RA}$ coeluted on Mono Q anion exchange chromatography with the type II cAMP-dependent protein kinase holoenzyme. An antiserum specific to RI, the cAMP-binding regulatory subunit of type I cAMP-dependent protein kinase, immunoprecipitated $p47^{RA}$. An antiserum specific to RII, the cAMP-binding regulatory subunit of type II cAMP-dependent protein kinase, immunoprecipitated $p51^{RA}$.

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 and fluorography as analytical tools.

Major Findings

1. **Retinoylation in HL60 Cells of the cAMP-Binding Regulatory Subunits of Type I and Type II cAMP-Dependent Protein Kinases.**
Retinoylation (retinoic acid acylation) is a posttranslational modification of proteins occurring in a variety of eukaryotic cell lines. There are at least 20 retinoylated proteins in the human myeloid leukemia cell line HL60 (Takahashi, N. and Breitman, T.R. (1990) J. Biol. Chem. 265, 19158-19162). Here we found that some retinoylated proteins may be cAMP-binding proteins. Five proteins,

covalently labeled by 8-azido-[^{32}P]cAMP which specifically reacts with the regulatory subunits of cAMP-dependent protein kinase, comigrated on two-dimensional polyacrylamide gel electrophoresis with retinoylated proteins of Mr 37,000 ($p37^{RA}$), 47,000 ($p47^{RA}$), and 51,000 ($p51^{RA}$) labeled by ^{3}H-retinoic acid treatment of intact cells. Furthermore, $p47^{RA}$ coeluted on Mono Q anion exchange chromatography with the type I cAMP-dependent protein kinase holoenzyme and $p51^{RA}$ coeluted on Mono Q anion exchange chromatography with the type II cAMP-dependent protein kinase holoenzyme. An antiserum specific to RI, the cAMP-binding regulatory subunit of type I cAMP-dependent protein kinase, immunoprecipitated $p47^{RA}$. An antiserum specific to RII, the cAMP-binding regulatory subunit of type II cAMP-dependent protein kinase, immunoprecipitated $p51^{RA}$. These results indicate that both the RI and RII regulatory subunits of cAMP-dependent protein kinase are retinoylated. Thus, an early event in RA-induced differentiation of HL60 cells may be the retinoylation of subpopulations of both RI and RII.

2. **Combinations of 24,24-difluoro-1α,25-dihydroxyvitamin D_3 and either Retinoic Acid, Sodium Butyrate, Dimethyl Sulfoxide, or Hexamethylene Bisacetamide Synergistically induce Monocytoid Differentiation of the Human Myeloid Leukemia Cell Line HL60.**

1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and some of its analogs are active differentiation agents *in vitro*. However, the use of these agents in the clinic has been hampered by serious complications arising from their effects on calcium metabolism. Here we investigated the interaction of the analog of 1,25-$(OH)_2D_3$, 24,24-difluoro-1,25-dihydroxyvitamin D_3 ($F_2(OH)_2D_3$), with either retinoic acid (RA), sodium butyrate (NaB), hexamethylene bisacetamide (HMBA) or dimethylsulfoxide (DMSO) on the induction of differentiation of HL60 cells. We analyzed these interactions with the Chou-Talalay median effect principle and found that combinations of $F_2(OH)_2D_3$ with either RA, NaB, DMSO, or HMBA synergistically induced differentiation of HL60 cells. These cells reduced nitroblue tetrazolium and were monocytes based on morphology and the expression of nonspecific esterase activity. In contrast, combinations of NaB and either HMBA or DMSO were antagonistic and combinations of HMBA and DMSO were additive for the induction of differentiation of HL60 cells. 1,25-$(OH)_2D_3$ was about 10-fold less potent than $F_2(OH)_2D_3$ in inducing monocytic differentiation of HL60 cells. However, combinations of 1,25-$(OH)_2D_3$ and RA synergistically induced differentiation of HL60 cells. These results may suggest that combinations of 1,25-$(OH)_2D_3$ and some of its analogs with either RA, NaB, or HMBA may be useful for clinical therapy of some leukemias.

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 other 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 and 1,25-dihydroxyvitamin D_3 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 see if fresh cells from leukemia patients respond *in vitro* to these combinations.

Publications.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07190-01 LBC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Regulation of Intercellular Junctions

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, M.D.	Visiting Fellow	LBC, NCI
	Eduardo Sainz, M.S.	Chemist	LBC, NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Biological Chemistry, DTP, DCT

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3	PROFESSIONAL: 3	OTHER: 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.)*

Intercellular junctions play an important role in the function, stability and integrity of epithelial tissues. Invasive behavior can be produced in cultured epithelia by disrupting intercellular junctions. An understanding of the mechanism(s) involved in stabilizing intercellular junctions may lead to the rational design of drugs with anti-invasive and anti-metastatic activities.

This past year, we developed criteria for assessing junctional stability in cultured epithelia, developed a quantitative assay that reflects junctional stability, and investigated whether particular junctions could be selectively stabilized in a Ca++-depleted environment. The significant results of this study were: (1) the demonstration that desmosomal junctions can be selectively stabilized by at least two different mechanisms; the first, a rapid biophysical response mechanism mediated by organic anions, and the second, a slow metabolic response dependent upon inhibition of mevalonate metabolism, which maintains the original positions of half-desmosomes on cell surfaces and allows the rapid reformation of junctions when effective Ca++ levels are restored; and (2) the discovery that desmosomal maturation may be an integral part of epithelial t tissue differentiation insofar as it involves the acquisition of mechanisms for controlling the stability of desmosomal junctions independently of other types of intercellular junctions.

Objectives

Our studies have addressed the following issues: (1) developing criteria for assessing junctional stability; (2) developing a quantitative assay that reflects junctional stability; (3) determining whether particular types of junctions can be selectively stabilized, and if so, by what mechanisms? ("stability" = complex stability, not stability vs. degradation).

Methods

Underlying strategy:

Cultured MDCK renal adenocarcinoma cells were used as a test system. These cells are particularly well-described in the cell biology literature. When transplanted to appropriate animal hosts, they form renal adenocarcinomas. Under usual culture conditions they exhibit properties of well-differentiated transporting epithelia whose cells are interconnected by adherens, occludens, gap and desmosomal junctions.

A simple quantitative assay system was developed that reflected the nonselective loss of junctional connections when cells were deprived of extracellular Ca++. (The assay is based on the resistance of confluent cultures to cell rounding in the presence of chelating agents). This assay was used to discover agents that protected against the loss of junctional connections. Immunofluorescent and electron microscopy techniques were employed to determine whether particular types of junctions were selectively stabilized. Finally, causal analysis was used to determine the nature of the correlation between the assay response and junctional stability.

Major Findings: Summary

These studies indicated that:

(1) mature desmosomal junctions can be selectively stabilized against Ca++ removal by organic anions of chain length less than 5 carbons;

(2) the effect is likely to be mediated by specific anion binding to and stabilization of protein complex(es) in the junctional region;

(3) a product of mevalonate metabolism is required for desmosome internalization following splitting of junctions by Ca++ depletion;

(4) this product may be an isoprenylated molecule whose function is to regulate the association of cytokeratin-type intermediate filaments with the desmosomal plaque;

(5) stabilization of desmosomal junctions and resistance to cell rounding are not causally related events, but appear to be correlated by a common mechanism of action of organic anions.

Description of Results, Experiments in Progress and Significance

1. Criteria used to assess junctional stability.

(a) ability to resist cell rounding by chelating agents (EDTA, EGTA, CDTA, NIA, HIMDA);
(b) ability to resist the development of refractile boundaries (visualized by phase contrast optics) between flattened cells in low Ca++ (5μM) medium;
(c) immunofluorescent localization of junctional components (uvomorulin - adherens junction, desmoplakin-desmosomal junction) at regions of cell-cell contact;
(d) ultrastructural morphology of gap, occludens, adherens, desmosomal junctions.

2. Quantitative assay system

Two to three day old confluent cultures of MDCK incubated in TBS (0.02 M Tris + 140 mM NaCl, pH 7.6)+/- chelators +/- organic anions, incubated for 1 hr at 37° C, then fixed, stained with SRB (Skehan 1991), and scored at defined positions across dish diameter for % round cells.

3. Mechanisms of stabilization

A. Organic anions
The protective effect was not exhibited in subconfluent cultures but increased progressively with culture age after day two of confluency; desmosomal junctions were selectively stabilized. The protective effect was specific for organic anions, varied inversely with chain length but did not correlate with hydrophobicity (partition coefficient in octanol/H2O, was concentration-dependent in the 10-100 mM range, and was independent of the method(s) used to deplete Ca++ (e.g. switching to a low Ca++ medium, Ca++-free buffer, or the use of various chelating agents). The effect occurred rapidly after addition of the organic anion to the culture. These results point to a specific ion effect - mediated by polar interactions - on the stability of protein-protein complexes uniquely associated with the desmosomal junction. The dependence of the effect on age of confluency together with the observation that desmosomes are present at the time confluency is first achieved suggest that a putative organic anion binding protein is not a required as a structural component of the desmosome but acts in a regulatory capacity.

B. Inhibition of mevalonate metabolism
The inhibition of production and/or conversion of mevalonate to isoprenoids can be expected to impair the synthesis of multiple end products of isoprenoid metabolism, including sterols, dolichols, ubiquinones, and isoprenylated proteins. Pretreatment of MDCK confluent monolayers with mevinolin, a competitive inhibitor of HMG CoA reductase, completely prevents EDTA-induced cell rounding and internalization of desmoplakin, but not the disappearance of uvomorulin from cell junctional areas, or the loss of morphologically identifiable adherens junctions. F-mevalonate, which blocks the utilization of mevalonate for

isoprenoid synthesis, is partially effective as a protective agent. The protective effect of mevinolin and F-mev is blocked by mevalonate (.1 mM and higher concentrations.).

Comparisons were made of the inhibitor effects of mevinolin, F-mevalonate, OH cholesterol and two synthetic OH cholesterol analogs on cell rounding in the presence of EDTA and incorporation of 2-14C-acetate into cholesterol (determined by HPLC). There was no correlation between the degree of inhibition of cholesterol synthesis and the degree of resistance to cell rounding.

Desmosomal junctions appeared to be ultrastructurally indistinguishable from normal in mevinolin-treated cells. However, when these cells were treated with EDTA, all of the desmosomal junctions were split and there were wide intercellular spaces between the previously-joined cells. Extending from each of the split desmosomes, which remained on the cell surface, were heavy bundles of intermediate filaments, presumably cytokeratin.

Although the acquisition of resistance to cell rounding requires preincubation of cells with mevinolin for 16-18 hours, upon removal of the drug, there is a rapid (2-3 hrs) reversal which is accelerated in the presence of cycloheximide.

These results are consistent with a mechanism of stabilization involving a relatively slow time-dependent loss of a covalently modified protein (e.g. via protein degradation). The absence of the modification in some way facilitates the interaction of intermediate filaments with desmosomal components.

4. Experiments in progress

In collaboration with Dr. Philip Skehan and Ms. Heidi Bokesch (FCRF-NIH), we are using fluorescence techniques combined with video microscopy to measure changes in intracellular Ca++ levels and subcellular localization in response to treatments that stabilize or labilize desmosomal junctions. We are also investigating the role of mevalonate in desmosome function using a variety of analytical techniques.

5. Significance

It is generally believed that the formation of adherens junctions is a prerequisite for the assembly of all other types of epithelial junctions, and that extracellular Ca++ is required for junctional stability. Our studies are important in the following respects:

(1) They demonstrate the existence of at least two types of compensatory mechanisms that may operate to maintain desmosomal junctions in the absence of effective stabilization by Ca++. The first, a rapid biophysical response mechanism, prevents desmosome splitting and internalization; and the second, a slow metabolic response, maintains the original positions of half-desmosomes on cell surfaces and allows the rapid reformation of junctions when effective Ca++ levels are restored.

(2) They suggest that desmosomal maturation may be an integral part of epithelial tissue differentiation insofar as it involves the acquisition of mechanisms for controlling the stability of desmosomal junctions independently of other types of intercellular junctions.

(3) The spectrum of organic anions that effectively stabilize desmosomes includes known components of extracellular fluids as well as intracellular metabolites such as pyruvate, lactate and glutarate. This suggests a possible relationship between cell metabolism and desmosomal stability.

(4) These findings are not restricted to MDCK cells but have been demonstrated in various in vitro epithelial tumors obtained from the NCI in vitro drug screen in Frederick.

ANNUAL REPORT OF THE BIOLOGICAL TESTING BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

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 maintain *in vitro* screening capacity at the current level 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. Develop in vivo models for intitial testing that:

1. Are amenable to a short "turn around time" for providing testing results.

2. Require less compound material.

3. Provide internally confirmable results.

4. Provide performance data that can be reliably utilized for more extensive testing.

G. Continue to develop in vivo models, e.g. orthotopic that have the potential for more accurately predicting clinical activity.

H. 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 20,000 tests.

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. In vivo lines are in development to support and correlate with in vitro models.

(c) Breast cell lines are in development. It appears that 5-6 lines will be developed that are amenable to current in vitro panel protocols. Correlating in vivo cell lines are in development.

2. In vivo

(a) Testing has been performed as indicated with a 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.

(d) Studies for the development of a model for much needed preliminary in vivo testing with less compound requirements and quick results are in progress. Very preliminary results are promising.

II. AIDS Area

A. *In vitro* screen

1. The AIDS *in vitro* screen is now operational at a level exceeding 40,000 tests annually.

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. DCT now provides funding based on DCT animal usage. Current price lists for DCT and other users reflect a savings of 35-45% over purchase prices from commercial vendors with superior quality control plus the provision of 20-30 rodent strains that are only available through this effort.

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 1991

PRIMARY RODENT CENTERS (3)	$3,374,597
Supply breeding nucleus for the animal program and athymic mice for drug evaluation.	
RODENT PRODUCTION CENTERS (1)	260,751
Large-scale production of nude mice under barrier controlled environment.	
DIAGNOSTIC & HISTOCOMPATIBILITY PROJECTS (7)	689,266
To monitor animal health and genetic integrity.	
DEVELOPMENT OF STANDARDS & GUIDELINES (1)	31,000
For animal care and breeding.	
QUALITY CONTROL AND MODEL DEVELOPMENT (1)	1,094,905
PRIMARY SCREENING OF HUMAN AIDS VIRUS	651,857
HUMAN TUMOR CELL LINE PROCUREMENT	
Breast (1)	177,827
Prostate (2)	599,987
FREDERICK CANCER RESEARCH AND DEVELOPMENT CENTER (2)	
Animal Production	3,595,056
CANCER RESEARCH AND SUPPORT PROJECTS	5,228,000
AIDS RESEARCH AND SUPPORT PROJECTS	6,432,000
TOTAL ESTIMATED COST	$22,135,246

The above figures do not reflect reimbursements from grantees, VRB, and other NCI Divisions that are credited to the contracts.

ANNUAL REPORT OF THE DRUG SYNTHESIS & CHEMISTRY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

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 begins with the acquisition of large numbers of compounds for screening, including both synthetics and natural products of diverse chemical and biological types. On this scale, acquisition is carried out through a network of scientific contacts, maintained on a worldwide basis. During this year, approximately 10,600 new compounds (600 pure, characterized natural products and 10,000 synthetic compounds) were acquired from 704 suppliers (of whom 390 were new to our program). Leads identified in the screens are optimized by the synthesis of congeners and prodrugs under a series of contracts from DS&CB. Unavailable literature compounds of interest as well as compounds needed for further evaluation are resynthesized on special contracts and task order agreements (93 compounds total in the current year). Other contracts are for synthesis of radiolabeled drugs, as required. E.g., labeled syntheses of 9-aminocamptothecin, 9-mthoxyellipticinium acetate, and Uniroyal Junior were completed in the current year, and labeled syntheses of taxol and Dolastatin 10 are in progress. NCI's repository of some half-million compounds is maintained by DS&CB for the storage and systematic distribution of samples within DTP, to other NIH programs, and upon request to investigators worldwide (2,500 compounds this year).

The flow of compounds into the cancer and AIDS screens is implemented by the Acquisition Input Committee, chaired by DS&CB. About 200 compounds weekly are being submitted to each screen. (The approximately 8,000 acquired compounds that accumulated during the development of the new cancer screen have all now been tested.) Novel, non-Discreet structures from the repository not previously submitted for the AIDS screen are being identified by computer cluster analysis, then selected manually for testing; several of these were active and at least one (NSC 287474) was selected for further study. Efforts to identify structure-activity relationships include computer-assisted comparison and analysis of the tests results. Novel leads generated from the AIDS screen include series of benzothiadiazenes (NSC 287474), dibenzocyclooctanes (NSC D634251), lexitropsins (NSC 634555), N-cyanothioureas (NSC D637937), and chalcones (NSC 636790). Efforts in anti-AIDS congener synthesis have produced active azostilbenes, thiosuramins, and monomeric aurintricarboxylic acids that have been selected for further study; active castanospermine derivatives have been obtained that do not depend on the common mechanism of glucosidase inhibition. Efforts in anti-cancer congener synthesis have produced active, CNS-specific analogues and prodrugs of the ellipticinium lead that may overcome its pharmacokinetic limitations. Lipophilic platinum complexes with greater potential for cell uptake have been synthesized. The synthesis of water soluble prodrugs of the modestly potent microtubule inhibitor Combretastatin-A4 is in progress. A new synthetic effort in optimization of natural product leads has just recently been initiated; as the first project, synthesis of an accessible topological model of the taxol structure is planned. DS&CB

collaborates with intramural scientists by synthesizing compounds for special projects, such as a recent series of intercalating one-armed mustards. DS&CB staff members also provide in-depth background searches and evaluation on compounds (over 200 so far) showing sufficient activity in the cancer screen to be considered for further development.

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 91

Contractor	Investigator	Contract No.
*Alabama, University of	Baker	N01-CM-87267
ERC BioServices Corp.	Groover	N01-CM-07317
*Georgia Tech Research Corp.	Zalkow	N01-CM-87269
**Illinois, University of	Farnsworth	N01-CM-87226
Japanese Foundation Cancer Res.	Tsukagoshi	N01-CM-17535
*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
SRI International	Dawson	N01-CM-17502
*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	Caine	N01-CM-07335
H.G. Pars Pharmaceutical Labs.	Pars	N01-CM-07339
Research Triangle Institute	Seltzman	N01-CM-07330
Ricerca, Inc.	Neal	N01-CM-07331
Southern Research Institute	Temple	N01-CM-07329
SRI International	Tanga	N01-CM-07333
Starks Associates	Hsiao	N01-CM-07341

*These contracts are being recompeted in the current year.
**This contract will be phased out June 29, 1991.

PUBLICATIONS:

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Cushman M, Chinnasamy P, Chakraborti AK, Juray J, Geahlen RL, Haugwitz RD. Synthesis of [ß-(4-pyridyl-1-oxide)-L-alamine[4]]-angiotensin I as a potential suicide substrate for protein-tyrosine kinases, Int J Pep Protein Res 1990;36:538-43.

Whaley R, Hodes L. Clustering a large number of compounds. 2. Using the connection machine, J Chem Inf Comput Sci 1991;31:345-7.

Hodes L, Feldman A. Clustering a large number of compounds. 3. The limits of classification, J Chem Inf Comput Sci 1991;31:347-50.

Lednicer D. Twenty five years of annual reports in medicinal chemistry, Annu Rept Med Chem 1990;25:333-40.

Narayanan VL. Development of new chemotherapeutic agents. In: Magrath I, ed. New Directions in Cancer Treatment. New York: Springer-Verlag, 1990;227-42.

Bader JP, McMahon JB, Schultz RJ, Narayanan VL, Pierce JB, Harrison WA, Weislow OS, Midelfort CF, Boyd MR. Discovery of a novel potent inhibitor of HIV infection. In: Kumar A, ed. Advances in Molecular Biology and Targeted Treatment for AIDS. New York: Plenum Press, 1991;309-313.

Lednicer D, Snader KM. Plants and other organisms as a source of anti-HIV drugs. In: Garnsworth N, ed. Economic and Medicinal Plant Research, Vol. 6. London: Academic Press. In press.

Bader JP, McMahon JB, Schultz RJ, Narayanan VL, Pierce JB, Harrison WA, Weislow OS, Midelfort CF, Stinson SF, Boyd MR. Oxathiin carboxanilide, a potent inhibitor of human immunodeficiency virus reproduction, Proc Natl Acad Sci USA. In press.

PATENTS:

Anderson WK, Haugwitz RD. US Patent 4,994,591: Platinum complexes derived from ß-silyamines, February 19, 1991.

Deutsch HM, Glinski J, Haugwitz RD, Narayanan V, Suffness M, Zalkow L. US Patent 4,942,184: Water soluble, antineoplastic derivatives of taxol, July 17, 1990.

*Baker DC, Hand ES, Haugwitz RD, Narayanan VL. US Patent 4,816,468: 5-Cyanopyridine-2-diazohydroxide, basic salts and use, March 28, 1989.

*This patent was inadvertently omitted from 1990 Annual Report.

ANNUAL REPORT OF THE GRANTS AND CONTRACTS OPERATIONS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

This Branch provides an administrative and managerial focal point for the support and coordination of DTP extramural activities involving 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 research project is investigator initiated; (2) the project is carried out without substantial NCI program involvement; and (3) there is no expectation by NCI for a specified service or product. The Biochemistry and Pharmacology Grant Program (BP) supports all aspects of anticancer drug discovery and development research. 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. Table 1 lists BP grants by topic subcategory. At the end of FY 1990, 315 grants totaling $64,642,000 were administered by the Branch (Table 2). This represents a drop from 336 in FY 1989. The reasons for the decline are complex and typify the situation throughout NIH. Congress has suggested changes in both the review and award of grants to increase the award rate by more emphasis on cost containment and a reduction in average award length. During FY 1991 a total of 318 grant applications were received. About 29.8% are expected to be awarded by the end of the fiscal year, which is higher than the 26.4% of all competing applications funded in FY 1990. During this transition time, BP has actively participated in an examination of funding mechanisms, such as Outstanding Investigator Grants and Program Project Grants, to ascertain if guidelines and requirements should be changed. BP also recommended several grants for James A. Shannon Director's Awards, an innovative mechanism instituted by the new NIH Director, Dr. Bernadine Healy, for grants which missed the payline but were near the margin of excellence. In making selections, special attention was paid to the Director's "Women's Health Research Initiative".

The Branch issues Requests for Applications (RFAs) to encourage research in underfunded areas of importance to the NCI mission. As a result of a Branch organized workshop on "Taxol and Taxus: Current and Future Perspectives", held June 26, 1990 in Bethesda, Maryland, an RFA was issued to stimulate novel approaches for solving the taxol supply problem and to support further study of this clinically active

agent. Sixty one applications were received and multiple awards are expected by the end of the fiscal year over a range of areas of taxol research: synthesis; tissue culture; plant genetics and propagation of Taxus; biosynthesis; binding to microtubules; analysis and assay development; metabolism; formulation of clinical dosage forms; and experimental and clinical therapeutic studies.

Several BP grantees made important scientific contributions during the past year, but some deserve special mention. William Plunkett, Ph.D. (CA28596) is continuing his studies on the cellular pharmacology and molecular mechanisms of action of arabinosylcytosine (ara-C), fludarabine (F-ara-A) and 2',2'-difluorodeoxycytidine (dFdC) to optimize their anticancer activity alone and in combinations. For example, he found that a combination of mitoxantrone and ara-C was effective only when the ara-C was given first. The effectiveness correlated with a significant increase in DNA strand breaks. This result provides a rationale for sequence-specific combinations of DNA damaging agents, such as mitoxantrone, with ara-C or other nucleoside analogs that inhibit DNA replication and repair. Such basic studies are providing information for the rational design of clinical combination treatment protocols and represent an outstanding example of "translational" research, i.e., applying laboratory findings to the clinical setting. George Weber, M.D., an Outstanding Investigator (CA42510), has contributed significant new information on the ability of tiazofurin, a selective blocker of inosine 5'-phosphate dehydrogenase activity, to down-regulate expression of the c-Ki-ras oncogene in a patient with chronic granulocytic leukemia in blast crisis, a disease where tiazofurin has some efficacy. This work, together with Weber's extensive studies on the metabolic activation and degradation of tiazofurin, suggests a rational, enzyme-targeted approach to chemotherapy, which has resulted in increased clinical interest in the drug. Len Erickson, Ph.D., (CA 45628) confirmed earlier observations by Drs. Dolan, Pegg and Moschel that O-6 benzylguanine in combination with BCNU produces depletion of O-6 methylguanine DNA methyltransferase (MGMT) in cultured HT-29 colon tumor cells. Additional in vivo studies on this combination are now being pursued as a prelude to potential clinical trials of the combination. In follow-up studies on the regulation of MGMT, a concerted effort has been initiated to study MGMT for restriction fragment length polymorphisms. Surprisingly, DNA from Mer+ cells, which contain high levels of MGMT and MGMT mRNA, were almost totally resistant to cleavage by Hpa II and Hha I, two restriction enzymes. By contrast, DNA in Mer- cells, which are completely devoid of MGMT protein and mRNA, was hypomethylated and cleaved by the restriction enzymes. The lack of cleavage in the Mer+ cells was due to hypermethylation, a pattern that is contrary to that seen in most other expressed genes described to date. This unusual finding is being actively pursued.

Contracts

The contract mechanism is used to procure resources or services needed 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 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, including multiple master agreement holders.

During FY 1991 an in depth review was made of the entire portfolio of DTP contracts to identify projects that could be effectively and economically combined over the next several years at the time of recompetition. This review has resulted in plans for the consolidation of large-scale preparation of both preclinical and clinical bulk drugs through a network of generic rather than separate contract packages. These combinations are being reflected in Requests for Proposals (RFPs), and will provide savings in costs and in staff efforts required to monitor the resulting contracts.

Table 3 identifies by title the solicitations and awards for FY 1991. The estimated total value of the 1991 awards over the anticipated period of each contract is $40,927,873. RFPs for 12 projects were issued during FY 1991, for FY 1992 award, at an estimated cost of $55,587,461 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 (NCDDG) 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 clinical efficacy. During FY 1990 the NCDDG was greatly expanded with the addition of eight new Groups emphasizing general mechanism of action and specific disease-oriented strategies and four more Natural Products

Drug Discovery Groups which are concentrating on the isolation of novel agents from natural sources. The new awards bring the total funding for FY 1990 to $13,456,780 for the support of 22 Groups (Table 4). During FY 1991 the Branch plans to reissue the RFA for NCDDGs to allow expiring incumbent Groups to recompete and to encourage the formation of new Groups so that total funds for the program will remain about the same. A Program Announcement to encourage model development is planned for FY 1991.

The NCDDG program has been successful in bringing several new therapies to clinical trial. Five agents reported previously remain under active clinical investigation through other support mechanisms. These include topotecan, an analog of camptothecin with improved water solubility that also forms a complex with topoisomerase I and DNA, which is in Phase II clinical trials; HomoDES, a potent inhibitor of polyamine biosynthesis which is in limited Phase I clinical trials at the University of Florida at Gainesville; monoclonal antibody 225, an IgG1 antibody which binds with high affinity to the human EGF (epidermal growth factor) receptor and blocks EGF-induced activation of tyrosine kinase; DAB486-IL-2, a novel diphtheria toxin-related interleukin-2 fusion protein which is being examined in patients with T-cell leukemias or lymphomas bearing high affinity interleukin-2 receptors; and 42/6, an IgA murine monoclonal antibody directed against the human transferrin receptor, which is the first IgA antibody to be evaluated in human trials. Two new IgG1 murine monoclonal antibodies, 27.15 and E2.3, developed by Dr. Ian Trowbridge, Salk Institute for Biological Studies, were recently approved by the NCI Decision Network for development to clinical trial. These antibodies, also directed against the human transferrin receptor, are proposed as a combination and represent the first attempt to use two antibodies directed against the same antigen target.

Based on the success of the NCDDG program in providing a framework for cooperation among several institutions while at the same time including the Government as a partner in the research, efforts are underway by Branch staff to expand this concept to the Small Business Innovation Research Program. In addition, Branch and other NCI staff are participating in discussions with several Government agencies, including the Agency for International Development and the National Science Foundation, to explore an NCDDG-like mechanism for use on an international scale to stimulate drug development and economic growth in the Developing Countries while at the same time strengthening global efforts to protect biological and cultural diversity.

Awards

A former BP grantee, Victor Ling, Ph.D., Ontario Cancer Institute, received a prestigious General Motors Award for his studies partially supported by BP on drug resistance. Matthew Suffness, a Branch Program Director, received the Employee of the Month award for organizing a workshop on taxol and for stimulating and coordinating research efforts within the grant program on this important new agent.

Publications

1. Powell RG, Plattner RD, Suffness M. Occurrence of sesbanimide in seeds of toxic Sesbania species, Weed Science 1990; 38:148-152.

2. Suffness M, Pezzuto JM. Assays related to cancer drug discovery. In: Hostettmann K. ed. Methods in Plant Biochemistry. vol 6. Assays for Bioactivity: Academic Press, London, 1991;71-133.

TABLE 1

BIOCHEMISTRY AND PHARMACOLOGY GRANTS

PROGRAM AWARDS BY SUB-CATEGORY

FISCAL YEAR 1990

A	Synthesis and Chemistry	74	$12,160,407
B	Natural Products	25	4,191,701
C	Screening and Experimental Therapeutics	35	4,528,787
D	Comparative Pharmacology	28	3,971,084
E	Other Preclinical Aspects	3	363,477
F	Mechanism of Action	117	20,962,843
	Program Projects	3	4,635,577
	TOTAL	285	$50,813,876

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

BIOCHEMISTRY AND PHARMACOLOGY GRANT PROGRAM
ANALYSIS BY MECHANISM
(Dollars in Thousands)

	Fiscal Year 1990	
	Number	Amount
Traditional (R01)	228	$35,863
Program Project (P01)	3	4,636
Conference, New Investigator and Small Grants	7	40
Outstanding Investigator	9	4,792
MERIT Awards	8	2,186
FIRST Awards	31	2,689
Cooperative Agreements	22	13,407
Small Business Innovation Research Awards	7	1,029
TOTAL	315	$64,642

TABLE 3

TOTAL CONTRACT VALUE (ISSUED/AWARDED)

ANTITUMOR PROJECTS

Master Agreements for Chemical Synthesis	$ 1,418,625
Synthesis of Congeners and Prodrugs	3,591,659
Maintenance of a Rodent Production Center	775,515
Operation of an Animal Diagnostic Laboratory	1,326,061
Synthesis of Bulk Chemicals and Drugs for Preclinical and Clinical Studies	5,122,690
Synthesis of Bulk Chemicals and Drugs for Preclinical and Clinical Studies by Small Business	4,929,601
Master Agreements for Mechanism of Action and Biochemical Pharmacology Studies of Antitumor Agents	4,144,224
Shelf Life Evaluation of Clinical Drugs	1,630,891
In Vivo Evaluation of Combination Therapy for Anticancer Activity	945,750
Total	$23,885,016

ANTI-AIDS PROJECTS

Preparation of Radiolabeled Anti-AIDS Compounds	2,183,068
Cultivation of Marine Protista	996,190
Large Scale Preparation of Anti-AIDS Bulk Drugs for Phase II and III Clinical Trials	2,895,090
Synthesis of Congeners and Prodrugs of Anti-AIDS Compounds	1,704,580
Plant Collection and Taxonomy of Shallow Water Marine Organisms	3,193,814
Detailed Drug Evaluation of Anti-AIDS Agents	1,576,250
Plant Collections and Taxonomy	3,315,379
Preparation of Anti-AIDS Bulk Drugs and Chemicals	5,509,054
Preparation of Anti-AIDS Bulk Drugs and Chemicals by Small Business	5,509,054
Plant Collection and Extraction in Brazil	472,875
Total	$27,355,354

TABLE 3, CONTINUED

ANTITUMOR AND ANTI-AIDS PROJECTS

Computer Based Searches for Chemical Structures	635,447
Natural Products Lead-Based Structures	1,761,678
Development and Production of Parenteral Dosage Forms for Clinical Studies	11,605,484
Resynthesis of Compounds for Screening	2,522,000
Analysis of Chemicals and Pharmaceutical Formulations	2,237,881
Development of Novel Drug Formulations and Delivery Systems for Anti-AIDS Agents	788,125
Analysis of Anticancer and Anti-AIDS Chemicals and Pharmaceutical Formulations	6,818,629
Dosage Forms and Delivery Systems for New Drugs	4,088,970
Primary Rodent Production Centers	10,718,500
Preclinical Pharmacology Studies of Antitumor and Anti-HIV Agents	4,098,250
Total	$45,274,964
GRAND TOTAL	* $96,515,334

* Includes $55,587,461 issued in 1991 for awards in FY 1992 and $40,927,873 awarded in FY 1991.

TABLE 4

NATIONAL COOPERATIVE DRUG DISCOVERY GROUP PROGRAM

TOTAL COSTS FOR FY 1990

GRANT NUMBER	INVESTIGATOR/INSTITUTE/TITLE	COSTS
5 U01 CA 45967-04	Brattain, Michael G. Baylor College of Medicine Growth Regulation of Human Colonic Neoplasms	$ 401,533
1 U01 CA 52857-01	Brem, Henry Johns Hopkins Hospital Controlled Release Polymers for Brain Tumors	$ 1,034,360
5 U01 CA 50743-02	Chang, Ching-Jer Purdue University Mechanism-Based Discovery of Antitumor Agents	$ 368,700
5 U01 CA 50750-02	Clardy, Jon C. Cornell University New Anticancer Drugs from Cultured and Collected Marine Organisms	$ 604,846
3 U01 CA 45962-03S1	Corbett, Thomas H. Wayne State University Drug Discovery--Anticancer Agents for Colorectal Cancer	$ 37,500
1 U01 CA 52956-01	Cordell, Geoffrey University of Illinois at Chicago Novel Strategies for Plant-Derived Anticancer Agents	$ 852,849
1 U01 CA 52955-01	Crews, Philip University of California Discovery of Antitumor Marine Products by Mechanism Based Approaches	$ 598,140
5 U01 CA 50771-02	Hecht, Sidney, M. University of Virginia Mechanism-Based Discovery of Novel Antitumor Agents	$ 502,399
1 U01 CA 51880-01	Houston, L. L. Cetus Corporation Engineered Antibreast Cancer Single-Chain FV Immunotoxin	$ 1,215,545

TABLE, 4 CONTINUED

5 U01 CA 46088-04	Johnston, Michael R. University of Colorado Health Science Center Targeted Therapy for Lung Cancer	$ 620,690
1 U01 CA 53617-01	Levin, Victor A. University of Texas Discovery and Development of Tyrosine Kinase Inhibitors	$ 576,634
1 U01 CA 51908-01	Lippman, Marc E. Lombardi Cancer Research Center Growth Regulation as Targets in Breast Cancer Treatment	$ 633,489
1 U01 CA 52020-01	McCaffrey, Ronald P. University Hospital Specific Therapy for TDT-Positive Leukemia/Lymphoma	$ 370,188
1 U01 CA 51992-01	McCormick, Frank P. Cetus Corporation Discovery of Drugs Inhibiting Oncogenic Ras Proteins	$ 866,110
2 U01 CA 37641-06	Mendelsohn, John Sloan-Kettering Institute for Cancer Research Anti-receptor Monoclonal Antibodies in Cancer Treatment	$ 794,986
5 U01 CA 48626-03	Murphy, John R. University Hospital Growth Factor Receptor Targeted Toxins for Leukemia	$ 604,120
5 U01 CA 37606-07	Porter, Carl W. Roswell Park Cancer Institute Inhibitors of Polyamine Biosynthesis and/or Function	$ 701,480
1 U01 CA 52995-01	Powis, Garth Mayo Foundation Cancer Drugs Active Against Signal Transduction Targets	$ 554,315

TABLE 4, CONTINUED

1 U01 CA 51946-01	Reisfeld, Ralph A. Scripps Clinic and Research Foundation New Tumor Models for the Development of Immunotherapy	$ 280,134
1 U01 CA 51958-01	Steplewski, Zenon S. The Wistar Institute Radioisotope-Antibody Conjugates for Cancer Therapy	$ 822,654
1 U01 CA 53001-01	Valeriote, Frederick Wayne State University Discovery of New Anticancer Agents from Natural Products	$ 757,331
5 U01 CA 48405-03	Wahl, Geoffrey M. The Salk Institute Detection and Curing of Amplified Genes in Human Cancer	$ 258,777
	TOTAL	$13,456,780

ANNUAL REPORT OF THE INFORMATION TECHNOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

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. ITB maintains similar databases for the DTP anti-AIDS screen initiated in mid-1987.

THE COMPARE DATA PATTERN MATCHING COMPUTER PROGRAM:

The data stored in the DTP anti-cancer and anti-AIDS databases are used initially to determine the extent of interest or lack thereof of the compounds tested. For the *in vitro* cancer screening data, one computer program used routinely to analyze the screening data is COMPARE.

COMPARE is an *in vitro* data pattern matching computer program designed and developed within DCT (an early version of COMPARE was described in J. Natl Cancer Inst, 81:1088-1092, 1989). It has a unique ability to determine the biochemical mechanism of action of a test agent using *in vitro* screening data. Thus, it can be used to find new agents having a specified mechanism of action.

Collaborations with DTP intramural investigators have been established to pursue leads discovered by COMPARE in the areas of tubulin binders, topoisomerase I or II, and antimetabolites. The first publication describing the discovery of a potent tubulin binder by COMPARE and the subsequent laboratory exploration of the tubulin binding activity was accepted by J. Biol. Chem. Scores of new structural types having tubulin binding activity have been discovered by COMPARE and the activity proven by DTP intramural investigators.

COMPARE easily detects agents which act through interaction with Topoisomerase I or II, but new structural classes having either of these activities appear to be rare. However, at least one new structural type of topoisomerase II agent has been discovered by COMPARE and confirmed by DTP intramural investigators. No new structural types having topoisomerase I activity have been confirmed as yet.

Several new potential antimetabolites suspected of inhibiting enzymes of de novo pyrimidine biosynthesis have been identified by

COMPARE. Laboratory investigations of these new agents are in progress.

This year COMPARE programs have been modified to use correlation coefficients as the key numeric relating seed patterns to database compounds' patterns. Previously, an average difference method was used. Both types of algorithms are currently available and give similar (though not identical) results. An option for the automatic printing of chemical structures in the order of the COMPARE listing is now available. The creation and automated weekly updating of databases for natural product extracts and other specialized databases has extended the range of applications of COMPARE. All available COMPARE programs are now available for the GI50, the TGI, and the LC50 levels of analysis. All COMPARE programs are now easily accessible from the DTPMENU user-friendly interface application system.

COMPUTERIZED SYSTEMS TO GENERATE AND DISTRIBUTE SUPPLIER REPORTS:

The data stored in the DTP databases are used also for creating reports to suppliers detailing the results of tests performed on their compounds. The systems required to successfully produce a flawless stream of high quality supplier reports are a great deal more numerous and more complicated than one might expect. ITB staff and contractors together have built a highly automated, very efficient, robust, and flexible supplier reporting systems for anticancer screening data and anti-AIDS screening data:

Cancer Supplier Reporting:

- Developed a new release of the Cancer Supplier Reporting (CSRS) system. The previous version was converted from a combination of SAS, C and DCL scripts to being written completely in the C language. This resulted in a much more robust program that is considerably more efficient than the previous program. The new system is more reliable, and much faster than it's predecessor. Several design criteria, such as open access and modularity, made the integration with the overall supplier reporting possible. The CSRS continues to be upgraded and refined.

- Designed and implemented a reporting system which supports the production and distribution of individually tailored subsets of screening data for review by the Screening Data Review Committee (SDRC), entry and automatic printing of SDRC decisions and comments on individual supplier reports, and the production and addressing of mailing copies of supplier reports. This system provides all essential audit trails to ensure that the supplier reports are reliably produced and distributed to all suppliers on a timely basis.

AIDS Supplier Reporting:

- Rewrote the AIDS DECISION MODULE (ADM) and the NATURAL PRODUCTS AIDS DECISION MODULE (NADM) applications in SQL*forms 3.0 and SQL*report-writer. This was done to take advantage of a standard method of code generation and to allow a common look and feel between all ITB applications. In rewriting these applications several errors in logic that had been causing problems in the old system were corrected. All historical data generated by the previous applications were checked and corrected.

- Developed a new release of the AIDS Supplier Reporting System (ASRS). The new system runs much faster and is more reliable and efficient than the previous version.

- Developed integration software between the ADM and the ASRS. The new system is so simple to operate that it has been turned over to the staff of the Antiviral Evaluation Branch who now can request and manage all AIDS supplier report production.

- Development of Aids database plate editing system for simplifying the identification and correction of erroneous data with full-screen interactive programs. It has been modified to show quality control codes and sort concentrations.

- Designed and developed a new percent protection-based calculation module for AIDS in vitro test results.

COMPUTERIZED IN VIVO ANTI_TUMOR TEST DATA SYSTEM:

The in vivo anti-tumor data system development was continued. In the previous year a basis was developed for a single model, the advanced stage subcutaneously implanted tumor model. With the enhancements added this year, the system now supports the following models:

1. Advanced stage subcutaneously implanted tumor.
2. Early stage subcutaneously implanted tumor.
3. Subrenal capsule implanted tumor.

The system design is extremely flexible and will accommodate other assay types, such as bioassay, non-tumored, and lifespan assays as the requirements arise.

The system provides data storage and retrieval, computations for the evaluation of drug effectiveness, and tabular and graphical presentation of raw and calculated data.

THE DTP COMPUTER CENTER AT FCRDC (DTP CC):

During 1990 it became clear that DTP computing requirements had very quickly outgrown the VAX 8820 and the 19 Gigabytes magnetic

disk capacity. Early in 1991, a very fast VAX 9210 was installed along with an additional 12 Gigabytes of disk space.

The DTP-CC completed an analysis of database size and growth. They concluded that current disk space should be sufficient for 2-3 years at current levels of data acquisition.

The DTP-CC also completed a thorough review and revision of the DTP-CC BACKUP procedures to assure safety and recoverability of the database in the event of some disastrous system crash. This study will be incorporated into a Federally-mandated disaster contingency plan which will be developed by ITB in collaboration with DTP-CC over the coming year.

The DTP-CC also completed a major revision of the architecture of the database this year.

MISCELLANEOUS NEW OR ENHANCED SYSTEMS:

- The DTP RETRIEVAL SYSTEM was designed, coded, loaded, and implemented for the DCT Director. This application creates an interface between general information on DN compounds stored in ORACLE tables and detailed information (including chemical structures) stored in Word Perfect files.

- The DTPMENU SYSTEM became available for general use. This menu lists essentially all available programs in the DTP system and permits the user to select and execute their selection from among them with a few keystrokes. On-line context-sensitive help is available, replacing the old instruction manual system.

- The P-24 assay data system. This computerized support for the AIDS secondary assay system measuring P-24 levels was completed this year. It provides complete support for the assay from initial stages through supplier reporting. It has been installed at the Southern Research Institute AIDS laboratory and at FCRDC laboratories.

- The Syncytial assay data system. The first phase of the computerized support for the AIDS secondary assay system, which quantifies syncytia formation due to HIV in the presence of test drugs, was completed this year.

- Three subsystems to manage LLDRD natural product crude extract fractionation (the V subsystem), manage in vitro combination testing (the Z subsystem), and special assay testing (the P subsystem).

- A Practices and Procedures manual was developed and tested. This document set standards and procedures for configuration management to be followed by all DTP software development contractors. An audit process was developed for verifying compliance with the guidelines.

- Modifications were made to the drug preparation system. These changes enabled technicians to modify assignments, resulting in less data needing manual verification.

- The DIS was modified to improved search functions, several functions for integration with other systems, such as ORACLE, and major improvements to the shipping software.

- System level documentation for the chemistry system of the DIS was delivered.

- The Pharmaceutical Data System (PDS) modifications which included: upgrading the PC subsystem to provide network support; provide the capability to ship from multiple lots; and enable receipt of partial shipments;

TRAINING CLASSES GIVEN BY ITB STAFF

- Introductory course on SQL*plus and the AIDs tables.

- Introductory courses on SQL*forms 3.0, Kermit, Compare and the NSC Text Retrieval System.

TRAINING CLASSES TAKEN BY ITB STAFF

- ULTRIX Commands and Utilities

- ULTRIX System Management and Administration

- VAX/VMS Systems Management I & II

- VAX/VMS System Performance Tuning

- Introduction to SAS 6.0

Publications:

Bai R, Paull KD, Herald CL, Pettit GR, Malspeis L, Hamel E. Halichondrin B and homohalichondrin B, antimitotic marine natural products which inhibit microtubule assembly by binding in the vinca domain of tublin: discovery of their mechanism of action by computer analysis of differential patterns of cytotoxicity and implications for microtubule perturbations attributed to the phosphatase inhibitor okadaic acid, J Biol Chem. In Press.

Hodes L, Paull KD, Koutsoukos A, Rubinstein L. Exploratory data analytic techniques to evaluate anticancer agents screened in a tissue culture panel, J Biopharm Statis. In Press.

ANNUAL REPORT OF THE NATURAL PRODUCTS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 - September 30, 1991

The Natural Products Branch (NPB) is responsible for coordinating programs directed at the discovery and development of novel natural product agents for the treatment of cancer and AIDS. 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 production of new agents for preclinical and clinical development.

Contracts for the collection of plants from tropical areas and shallow-water marine organisms from the Indo-Pacific region have been in progress since September, 1986, and are currently being recompeted for another five-year period. A five-year contract for the cultivation of cyanobacteria will be completed in September, 1991, while a three-year contract for the cultivation of marine protozoa is due for completion in April, 1992, and will be recompeted for another three-year period. A project for the cultivation of unusual fungi was initiated at the Frederick Cancer Research and Development Center (FCRDC) in March, 1990, and a contract for the cultivation of marine anaerobic bacteria was awarded as a subcontract through the FCRDC contractor, Program Resources, Inc., in May 1991.

Over 31,000 extracts have been submitted for anti-HIV testing, and a number of *in vitro* active products have been isolated from plant and microbial extracts by chemists of the Laboratory for Drug Discovery Research and Development (LDDRD). Over 6,000 extracts have been tested in the human cancer cell line screen; a number of these have exhibited selective cytotoxicity and are being fractionated by LDDRD chemists. Considerable effort has been devoted to the large-scale production of taxol and the development of renewable sources of the drug, in collaboration with the NCI CRADA partner, Bristol-Myers Squibb, and the USDA.

Organization and Staffing

The Branch is organized into four functional segments which are: (1) discovery of new agents from microbial derived extracts; (2) discovery of new agents from plant extracts; (3) discovery of new agents from extracts of marine organisms; and (4) large-scale production 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 four professionals and two secretaries. The contracts managed by the Branch are outlined in Table 1.

Table 1.

Natural Products Branch Contracts

Contractor	Investigator	Contract No.	Contract Program Area
		Cancer	
Australian Institute of Marine Science	Murphy/Baker	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
Hauser Chemical Research	Daughenbaugh	NCI-CM9-7554	Large-scale Isolation of Antitumor Agents
Polysciences, Inc.	Sims	NCI-CM9-7621	Large-scale Isolation of Antitumor Agents

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-CMO-7345	Plant Recollections
Missouri Botanical Garden	Forero	NCI-CMO-7346	Plant Recollections
New York Botanical Garden	Balick/Daly	NCI-CMO-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
Hauser Chemical Research	Daughenbaugh	NCI-CM8-7272	Large-scale Isolation of Anti-HIV Agents
Polysciences, Inc.	Sims	NCI-CM8-7271	Large-scale Isolation of Anti-HIV Agents

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 have been 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, unusual fungi, such as mycobionts and basidiomycetes, are being collected from unique habitats worldwide, and 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 has been 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 Southeast 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 contact has been made with shamans and traditional healers in certain areas who are guiding the collection of medicinal plants. To date, over 22,000 samples have been received of which over 13,800 have been extracted to yield over 27,600 extracts. These contracts are currently being recompeted for a further five-year period. In addition, a Request for Proposals (RFP) for the investigation of Brazilian medicinal plants in collaboration with Brazilian research organizations is due to be issued in the near future.

Two novel alkaloids exhibiting *in vitro* anti-HIV activity have been isolated from a Cameroon plant by LDDRD chemists and are being considered for further development.

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 3,900 are on hand in the FCRDC Repository. Over 4,400 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.

Collaborations and Agreements

Collaborations have been established with Kunming Institute of Botany and the Natural Products Research Institute of Seoul University in the study of medicinal plants from China and Korea respectively. A collaboration with Dr. Paul Cox of Brigham Young University in the study of Polynesian medicinal plants has resulted in the isolation of potential anti-HIV agent from a plant used in Samoan traditional medicine. Other collaborations are with scientists of the Institute for the Study of Medicinal Plants of the University of Yaounde in Cameroon, and Tel Aviv University in the study of Red Sea marine organisms.

In performing plant and marine organism collections in over twenty countries worldwide, NCI has recognized the need to collaborate with organizations in these countries in the study of their natural resources. Branch staff have played a major role in formulating a Letter of Intent stating NCI's willingness to collaborate in such studies, and to compensate countries in the event of development of a new drug from an organism collected within their borders. This document has been well-received by scientists in both developed and developing countries, and Branch staff have discussed its implications at meetings in the U.S. and abroad, including international conferences in Kunming and Costa Rica. An important agreement to collect plants in Madagascar was recently signed between NCI and Madagascar authorities.

A workshop on "Drug Discovery, Biological Diversity, and Economic Growth" was jointly sponsored with the U.S. Agency for International Development, the National Science Foundation, and the Fogarty International Center in March, 1991. Representatives attended from developing countries, the research community and the pharmaceutical industry. Issues relating to collaboration with developing countries were discussed, and an interagency working group is now developing a strategy to coordinate their activities in promoting such collaborations.

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 anti-HIV screens; samples of these extracts will also be held for testing in later screens as they are developed. A number of research organizations have expressed an interest in testing the extracts in a variety of screens, and a policy which will govern the distribution of selected extracts to suitably qualified organizations is currently being formulated. 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, 1991

	Organisms Received	Organisms Extracted	Extracts Available	Extracts Submitted for Anti-HIV Testing	Extracts Submitted for Anticancer Testing
Marine Invertebrates	3,924	2,220	4,453	3,652	420
Cyanobacteria and Fungi	1,895	1,895	16,650	12,968	4,765
Terrestrial Plants	22,184	13,536	27,072	13,777	1,003
Marine Plants	892	644	1,288	508	82
Lichens	367	353	706	691	

Large-Scale Production Program

The development of taxol as an effective treatment for refractory ovarian cancer, as well as the observation of its activity against several other cancers, has resulted in escalating demands for the drug. Branch staff have promoted interactions between Hauser Northwest, the collection organization representing Bristol-Myers Squibb (BMS) (the NCI CRADA partner), and the United States Department of Agriculture Forest Service (USDAFS), thereby facilitating *Taxus brevifolia* bark collections in the Pacific northwest, including Idaho. Branch staff have also been instrumental in establishing contacts with the British Columbia Ministry of Forests, and bark collections are now starting in that region. In addition contacts have been established in eastern Canada (Quebec), China, India, Mexico and the Soviet Union and surveys of various *Taxus* species in those countries are under way.

For some time program staff have recognized the need to establish alternative renewable sources to replace the bark as the major source of taxol, and Branch staff have promoted interaction between BMS and groups investigating Indian sources, as well interactions with U.S. nurseries and a large lumber company in studying the potential of high-yielding *Taxus* cultivars. An extensive survey of over 500 *T. brevifolia* trees from all regions of the Pacific northwest is ongoing in collaboration with the USDAFS to identify high-yielding phenotypes for cultivation and use as stock for reforestation projects.

With various camptothecin derivatives showing some efficacy in early clinical trials, a collaboration is being initiated with USDAFS to cultivate *Camptotheca acuminata*. Supplies of camptothecin are currently available from China and India, but increasing demands could exceed those supply sources, and the cultivation project is regarded as an important backup source of this starting material.

In March, 1991, Branch staff organized a workshop on the large-scale production of natural products. Over 30 experts involved in various areas of natural product research and development attended, and a strategy for the scale-up of natural product production capability to cater for the various phases of preclinical and clinical development was recommended.

Publications:

Cragg GM, Snader KM. Taxol: The Supply Issue. Cancer Cells 1991;3:233-235.

Pettit GR, Cragg GM, Singh SB, Duke JA, Doubek DL. Antineoplastic Agents, 162. *Zephyranthes candida*. J Nat Prod 1990;53:176-178.

Pettit GR, Herald DL, Cragg GM, Rideout JA, Brown P. Antineoplastic Agents, 178. Isolation and structure of Lychnostatins 1 and 2 from South American *Lychnophora antillana*. J Nat Prod 1990;53:382-390.

Pettit GR, Numata A, Takemura T, Ode RH, Narula AS, Schmidt JM, Cragg GM, and Pase CP. Antineoplastic Agents, 107. Isolation of Acteoside and Isoacteoside from *Castilleja linariaefolia*. J Nat Prod 1990;53:456-458.

Witherup KM, Look SA, Stasko MW, Ghiorzi TJ, Muschik GM, Cragg GM. *Taxus* spp. needles contain amounts of taxol comparable to the bark of *Taxus brevifolia*: Analysis and isolation. J Nat Prod 1990;53:1249-1255.

ANNUAL REPORT OF THE PHARMACOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The Pharmacology Branch (PB) is involved with several aspects of preclinical drug development in both cancer and AIDS. Through contracts currently in place, detailed therapeutic studies are conducted using murine models and pharmacology studies are done concentrating primarily on analytical method development and pharmacokinetic studies in mice. During this year two additional concepts were developed and approved by the DCT Board of Scientific Counselors: (1) a master agreement contract dealing with the mechanisms of action of cancer agents (2) a contract dealing with preclinical therapeutic strategies of combinations of cancer agents.

The staff of this branch as well as serving as project officers on these contracts also serve on several DTP drug development committees: Drug Aquisition, Working Groups, Operating DTP, NIAID Animal Models, Decision Network DCT. A large effort by one staff member went into organizing the DTP Biological Evaluation Committee for cancer. This effort involved assembling the committee, assessing computerization needs and initiating formats for group needs and chairing the committee. Since July 1990, 498 agents have been referred to this committee by the In Vitro Screening Review Committee of DTP.

For detailed therapeutic studies with cancer drugs, advanced stage tumors, metastatic disease and drug resistant tumors in both human xenografts and murine tumor models are currently being used. For detailed therapeutic studies with AIDS drugs, murine retroviruses (Rauscher leukemia - R-MuLV and LP-BM5) are the current useful models. A new lentivirus, bovine immunodeficiency - like virus (BIV), was used successfully in a syncytium reduction assay to evaluate agents in vitro. Dutch Belted and New Zealand rabbits are being used in a concurrent BIV in vivo model. BIV carries genomic characteristics (rev, tat, and riv) similar to HIV-1.

Separate contracts are used to develop methods of analysis for antitumor and anti-AIDS agents of interest to the DTP program and to conduct detailed pharmacokinetic studies in order to better understand agents' in vivo behavior. These contracts were consolidated this year so that in the future they will be concept reviewed and awarded as a group.

Numerous cancer drug candidates were evaluated under the detailed therapeutic studies contract. A major effort entailed the evaluation of several camptothecins. Emphasis was placed on various formulations of 9-aminocamptothecin (9-AC) in the advanced stage HT-29 colon tumor xenograft. Other agents evaluated during this period include the camptothecin analogue 9-glycinamidocamptothecin, ellipticinium derivatives, a cholera toxin, dolastatin 10, Dr. Liotta's CAI, Upjohn's 1065 analogue NSC 615291D, other topoisomerase I and II inhibitors and Taxol for cross-resistance profile determinations. Drug combination studies were also conducted with Adriamycin/Taxol in vitro and O^6-benzylguanine/BCNU in vivo. Compunds of interest to the BEC committee were also studied: NSCs 626490 and 618857.

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. Three sulfated dyes (NSCs 631567D, 634747D, and 638352D) and Uniroyal Jr. were tested in vitro. Activity of AZT and ddC were used to evaluate the BIV syncytium reduction assay, with successful results. The in vivo experiments with BIV in rabbits are in a development stage. This model does appear to be successful with respect to positive viral takes in rabbits.

During this year three AIDS pharmacology contracts and three cancer pharmacology contracts continue in operation. Eight novel compounds with demonstrated in vitro anti-HIV activity were studied during this period on the AIDS contracts. Detailed pharmacokinetic investigations in mice were completed for Uniroyal Jr. (NSC 629243) in both hamsters and mice. Studies have indicated that the oral route of administration in sesame oil results in sustained therapeutic plasma levels. Efforts are continuing with this agent to identify metabolites. An analogue of this agent, NSC 634947D underwent method development and preliminary pharmacokinetics. Studies with NSC 640441D, a protease inhibitor were started, methods development and preliminary pharmacokinetics, are ongoing. Method development for a rhodium complex, NSC 619179D resulted in the development of an atomic absorption spectroscopy technique with good sensitivity. An HPLC technique was developed and preliminary pharmacokinetic data were collected on a thiazolobenzimidazole (NSC 625487D). Oral bioavailability of this agent is poor in an emulphor formulation. Additional work was conducted with an anti-sense oligonucleotide (NSC 624958), characterizing aspects of plasma protein binding, subcutaneous infusions, tissue distribution and subcellular fractionation. Method development and preliminary pharmacokinetics were also conducted with two additional compounds, stilbene aldehyde(NSC 618121) and a diarylsulfone (NSC 624231).

The cancer pharmacology contracts concentrated on several areas of interest to DTP. Pharmacokinetic studies in mice via various

routes of admininistration with different formulations of 9-amino-20(S)camptothecin (NSC 603071), (9-NH_2-CPT), in coordination with detailed therapeutic studies provided imformation on effective plasma concentrations and exposure times. These data provide targets for subsequent toxicology protocols. Of primary importance was the development of a unique HPLC postcolumn acidification technique to enable the measurement of the lactone and open ring forms of 9-NH_2-CPT. Pharmacokinetic studies in mice were conducted on the potentially useful prodrug 20-O-(N,N-dimethyl)glycinate dihydrochloride (NSC 639176). Several new ellipticine analogues had methods developed and were studied in attempts to determine their in vitro and in vivo stabilities. These were NSC 352299, the 9-unsubstituted OMS salt; NSC 639364, 9-Methyl acetate; and NSC 632855, the 9-Cl acetate. Cholera toxin pharmacokinetic studies in mice were completed and plasma concentrations and exposures at maximal tolerable doses stimulated additional in vitro scheduling experiments.

The work with method development of tetraplatin NSC 363812 is nearing resolution. Two important findings: tetraplatin itself breaks down rapidly in plasma to Pt(II)(Cl)2(dach) and this can be quantitated by HPLC and atomic absorption spectroscopy of Pt; processing of blood and plasma samples is crucial since tetraplatin can be lost by slow breakdown and plasma protein binding occurs with some breakdown products as well as with parent compound. Method development and pharmacokinetics was completed on the agent O^6-benzylguanine in mice and work continues on this agents effect on alkylguanine transferase in tissues and tumors.

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ANNUAL REPORT OF THE TOXICOLOGY BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The mission of the Developmental Therapeutics Program centers on the discovery and preclinical development of agents with anticancer or anti-AIDS potential. Investigations 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 the preliminary 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:

- Determine end-organ toxicities and additionally establish the dose-responsiveness and schedule-dependency of toxicity;
- Determine plasma drug concentrations and correlate levels to safety, toxicity and in vitro or in vivo efficacy;
- Determine the reversibility of toxicity during a post-dosing recovery period;
- Establish a safe starting dose for the Phase I clinical trials in humans.

This year three new antineoplastic drugs or analogs and four new anti-HIV agents moved into the IIA phase of preclinical toxicology. One of the anticancer drugs evaluated last year (Merck L651582) moved from IIA to IIB and another (9-amino[20S] camptothecin) is expected to do so shortly.

Of the five AIDS drugs that were in IIA last year, one was dropped in favor of a second generation analog (oxathiin carboxanilide), a second was dropped for other reasons and the other three remained under evaluation at the IIA level.

Of the three cancer drugs currently at the IIB level, one of these (penclomedine) completed evaluation and will move to Stage III shortly. 8-chloro cyclic AMP is still undergoing evaluation to determine if kidney toxicity can be ameliorated by changing the schedule. Evaluation of the Merck L651582 compound is also expected to be completed shortly.

One AIDS drug moved from IIA to IIB this year, the second generation Uniroyal (Jr.) compound NSC-629243.

In addition to studies on these drugs in preclinical development, studies on three other drugs already in clinical trials were initiated last year and will be completed shortly. 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 to determine if oral administration of this drug is possible. A fourth drug, Fostriecin will have pharmacokinetics in mice determined so that the drug will be able to undergo pharmacolically-guided dose escalation when the Phase I trial begins later this year.

DRUGS IN DN STAGE IIA ON WHICH PRELIMINARY TOXICITY STUDIES WERE PERFORMED

ANTICANCER

9-AMINO [20S]CAMPTOTHECIN	603071
MERCK L651582	609974[a]
DISCREET	615291D
CHOLERATOXIN	629801
DISCREET	630176D

ANTI-AIDS

OXATHIIN CARBOXANILIDE	615985[b]
RHODIUM COMPLEX	619179
DISCREET	619858D
TERBIUM POLYOXOMETALATE	622102
DIARYLSULFONE	624231
DIARYLSULFONE	627708
DISCREET	625487D
DISCREET	640441D

DRUGS IN DN STAGE IIB ON WHICH FULL-SCALE TOXICITY STUDIES WERE PERFORMED

ANTICANCER

8-CHLORO CYCLIC AMP	614491

ANTI-AIDS

UNIROYAL [JR] ANALOG	629243

DRUGS THAT COMPLETED EVALUATION IN DN STAGE IIB AND MOVED INTO STAGE III

ANTICANCER

PENCLOMEDINE	338720

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
FOSTRIECIN	339638

[a] MOVED TO STAGE IIB.
[b] DROPPED IN FAVOR OF A MORE PROMISING ANALOG.

The information generated from the toxicology and pharmacology studies, particularly those carried out under Stage IIB, comprise the major portion of the valuable 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 master toxicity study protocols for potential anticancer and anti-AIDS drugs. These protocols set forth jointly agreed to procedures for animal toxicity studies of antineoplastic anti-AIDS 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. Many of the protocols on file were completely revised this year to reflect current practice. 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 1991, the Branch continued an innovative project designed to provide more definitive information on the availability of anti-AIDS drugs in the serum from animals dosed with promising new AIDS therapies. The results to date show that 12/18 drugs active in vitro were also active in vivo indicating that effective concentrations of each drug are achievable in an animal model. The reasons for the lack of correlation with the other six are being investigated. Four other drugs were both negative in vitro and in vivo.

These preclinical studies enable the program to evaluate animal toxicity and potential human toxicity of compounds in early development as well as to evaluate the pharmacokinetic and metabolic characteristics prior to committing the program to full scale drug development.

Publications:

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ANNUAL REPORT OF THE PHARMACEUTICAL RESOURCES BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The Pharmaceutical Resources Branch (PRB) provides comprehensive pharmaceutical services to the various Programs of the Division of Cancer Treatment. The primary objective of the Branch is to supply high quality chemical substances and formulated products in support of preclinical and clinical drug development. This objective is accomplished essentially through contract support activities. During this report period, the Branch supervised a total of 28 contracts with a combined annual budget of approximately ten million dollars.

The major contract areas include: chemical preparation 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 and two secretarial personnel. The classification of the senior professionals is as follows: three PHS Commissioned Corps pharmacists, one Pharm.D. pharmacist, one Ph.D. analytical chemist, and two Ph.D. medicinal chemists.

Functional Areas:

Chemical Resources

The Chemical Resources area provides 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 against cancer or AIDS. Chemical Resources supervises twelve chemical prep lab contracts consisting of a combined annual effort of approximately 30 staff 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's) 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 area provides for: (1) analytical methods development and characterization of new investigational agents; (2) analytical assessment of chemical and formulated products for quality control release. The analytical service supervises contractors engaged in the development of analytical methodology to determine the purity of chemicals, content 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 as acceptance criteria for additional supplies, either from commercial sources or chemical preparation contractors. The specifications and validated analytical methodology are documented in a format suitable for submission to the Food and Drug Administration as part of the NCI's Investigational New Drug Applications. The contractors are 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 characterize structurally diverse groups of chemicals, and are also responsible for the development and application of stability-indicating methods for all new drug substances.

Analytical data developed on new investigational compounds are 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 area of PRB is responsible for conception and development of dosage forms suitable for clinical use in chemotherapy and AIDS programs. More than one-half of the drugs requiring intravenous delivery do not

exhibit adequate aqueous solubility or stability, and some form of pharmaceutical intervention is required. Standard approaches (salts, non-aqueous 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, activity in rodent models whenever possible, and feasibility for manufacture on production scale.

The Product Development service is responsible for the supervision and management of three pharmaceutical R & D contracts with a combined annual effort of about eight staff years.

Pharmaceutical Acquisition and Production

The Pharmaceutical Acquisition and Production area manages five pharmaceutical contracts with capabilities to produce a broad range of pharmaceutical products. The area 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 five staff year effort.

In addition, this area 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 3 million dollars. The area also coordinates the receipt and quality review of numerous drug products provided by pharmaceutical manufacturers in support of DCT-sponsored clinical trials.

The contractors managed by the Pharmaceutical Acquisition and Production area produced over 500,000 injectable units, and about 50,000 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 (NCI Investigational Drugs: Pharmaceutical Data 1990, NIH Publication No. 91-2141) which is updated periodically.

Goals and Accomplishments

During the past year the Branch has been actively involved in the development of emulsions and microdispersions for intravenous administration. Several agents have been formulated in mixed solvents for intravenous administration.

The Branch continued to be responsible for manufacturing significant quantities of formulated supplies of taxol for clinical trials. This involved major efforts of both the pharmaceutical contractors and staff for quick response to increasing demands of drug while anticipating supplies from the commercial sponsor.

We anticipate four to six new agents each from the AIDS and cancer screens will be advanced 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 clinical trials.

The PRB and the storage and distribution contractor have undertaken an important project requiring patient-specific drug distribution for two large studies sponsored by the National Surgical Adjuvant Breast and Bowel Project (NSABP B-23 and B-24) of tamoxifen vs. placebo in women with breast cancer.

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 shipping 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 microparticles for intravenous use.

Publications and Patents by Staff

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2. Silverton JV, Quinn FR, Haugwitz RD. Structure of isopropyl 2-chloro-5-(2-methyl-1,4-oxathiin-3-ylcarboxyamino)phenylcarboxylate, oxathiin carboxanilide, Acta Crystallogr, in press.

3. Greene RF, Collins JM. Effects of leucovorin on idoxuridine cytotoxicity and DNA incorporation, Cancer Res 1990; 50:6652-56.

4. Kaskar B, Heise L, Michalak RS, Vishnuvajjala BR. A convenient large scale Synthesis of protected D-ribonolactone from D-ribose, Synthesis 1990;11:1031-2.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CM 07183-05 PRB

PERIOD COVERED
October 1, 1990 - September 30, 1991

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 oxanthiin carboxanilide has been determined. The structure of cephalomannine is being investigated. Strain energies and quantum calculations are being carried out on these compounds.

ANNUAL REPORT OF THE ANTIVIRAL EVALUATIONS BRANCH

DEVELOPMENTAL THERAPEUTICS PROGRAM

DIVISION OF CANCER TREATMENT

October 1, 1990 to September 30, 1991

The primary responsibility of the Antiviral Evaluations Branch (AEB) is the supervision of the AIDS antiviral screen. This responsibility includes participation in the general and specific technical features of laboratory procedures, arranging for the efficient transfer of data, monitoring the screen for efficacy, sensitivity, and consistency, evaluating the results to determine activity, and transmitting results to suppliers and appropriate DTP staff.

This is the first full year of the existence of the AEB. Supervision of the screen previously was performed as an extension of the Office of the Associate Director, DTP, but the added assumed responsibilities and increased activities suggested that these functions would be best served in the branch format. Screening is performed in laboratories under contract at the Frederick Cancer Research and Development Center (Program Resource, Inc.) and in Birmingham, Alabama (Southern Research Institute). The screen examines the anti-HIV activity of synthetic compounds and purified natural products, acquired through the Drug Synthesis and Chemistry Branch, and crude extracts of biological materials acquired through the Natural Products Branch. The screen also is used in the selection of active fractions from biological extracts separated in the DTP Laboratory of Drug Discovery Research and Development, leading to the purification of active natural products.

Changes in the technical features of the screen have improved the efficiency and reliability of the antiviral tests. During the past year over 95% of all tests passed strict quality control standards. As a result, the real capacity of the screen has increased, and over 20,000 unique synthetic compounds and biological extracts were tested in the past year. Also, close attention to the amount of infectious virus employed in the tests has expanded the sensitivity of the screen to detect materials active at any stage of the HIV reproductive cycle.

Active compounds of unique structure or potent antiviral activity are examined in more detail. Inhibition of virus reproductive activity and protection of cells from HIV killing are confirmed by other tests. The range of action is examined by testing these compounds against several HIV-1 strains, HIV-2, and a murine retrovirus. Methods for testing such compounds against AZT-resistant HIV strains, and in combination with AZT are under development. Also, these compounds are tested in several T cell lines, fresh peripheral blood leukocytes, and possibly other cells. Collaborations have been arranged among the screening labs, NCI intramural labs, and other local research laboratories focusing on the mechanism of action of new active compounds, in order to define the stage of the virus reproductive cycle affected by the active compound.

Over fifteen classes of compounds not previously demonstrated to have antiviral activity have been discovered to inhibit HIV in the screen. One of these, oxathiin carboxanilide (NSC 615985) and a more potent analog (NSC 629243), was shown to interfere with virus reproduction at a stage after virus binding to cellular receptor but prior to reverse transcription. More specific studies showed that these compounds inhibit the fusions that occur when cells expressing the viral envelope glycoprotein are mixed with cells containing the viral CD4 receptor. This result indicates that the fusion of virion and cellular membranes required during an early stage of infection is prevented by oxathiin carboxanilide. Several other active compounds with differing modes of action currently are under examination.

Presentations on the AIDS antiviral screening program have been made at several local, national, and international meetings, including the symposium on Advances in Molecular Biology and Targeted Treatment of AIDS in Washington, D. C., the International Conference on Antiviral Research in Brussels, Belgium, the International Conference on the Biotechnology of Microbial Products in Sarasota, Florida, and the International AIDS Conference in Florence, Italy.

Publications:

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