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NOTICE OF INTRAMURAL RESEARCH PROJECT

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PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Biochemical pharmacology of antiretroviral nucleoside therapy for AIDS

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

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COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Associate Director, Clinical Oncology Program

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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 the past year a number of strategies for treating retroviral infections has emerged. It is now appreciated that every step in the life cycle of pathogenic human retroviruses could theoretically serve as a target for antiviral therapy. In particular, the process of reverse transcription appears to be an attractive target with practical results already at hand. A number of drugs and pharmacologic principles relevant to the administration of such drugs have been identified in the Clinical Oncology Program for application in the therapy of diseases mediated by pathogenic human retroviruses. One family of such drugs (dideoxy-nucleoside analogues) shows special promise. These drugs are now being administered to patients with AIDS and its related disorders. One drug in the family, AZT, has already reached prescription drug status.

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

Introduction

The treatment of pathogenic human retroviral infections is a formidable challenge. The challenge has taken on a new urgency since the advent of the acquired immunodeficiency syndrome (AIDS), which has become a major public health problem after its recognition as a new clinical entity in 1981. This disease is caused by the third known human T-lymphotropic virus (HIV) which replicates within critical cells of the immune system (particularly T cells and macrophages), leading to loss of CD4+ (helper-inducer) T cells and profound immunosuppression. This retrovirus has several other names including lymphadenopathy associated virus (LAV) and AIDS-related virus (ARV). It has recently been proposed by the human retroviral subcommittee of the International Committee on the Taxonomy of Viruses that the retrovirus which causes AIDS be called human immunodeficiency virus (HIV), and in the future this will become the general designation for this category of retrovirus. There have now been well over 30,000 cases of AIDS reported in the United States, and the disease is seen throughout the world. It has become evident that there may be a long prodromal period before the development of fulminant AIDS. Over one million people in the USA are now infected with HIV and this number is likely to increase. Perhaps 30% or more of infected persons may develop AIDS in a 3 to 5 year period of time so that even if the spread of HIV infection slows, the number of cases of AIDS is expected to continue to increase. While AIDS is an exceedingly important priority in its own right, what we learn about treating the retrovirus which causes AIDS could have broad implications or other medical and veterinary illnesses. These two factors taken together have made this disease the highest priority in Dr. Broder's group.

Rationale for the use of Anti-viral Therapy in AIDS

The formal proof by Gallo and his co-workers in 1984 that HIV was the causative agent of AIDS permitted for the first time the consideration of specific anti-retroviral therapy and, in particular, made it possible to develop therapies aimed at inhibiting the replication of the retrovirus which causes this disorder. It is worth noting that anti-retroviral therapy for the treatment of AIDS is based on the assumption that continued retroviral replication is involved in both the pathogenesis and progression of this disease. As we shall discuss, this assumption has been amply validated. In regard to the immunodeficiency, the hallmark depletion of CD4+ T cells can be mediated directly by HIV infection of these cells although it is possible that indirect mechanisms such as auto-immune reactions or the production of toxic lymphokines may also play a role. If the immune system of these patients retains some regenerative potential (either alone or with the addition of immunoreconstitutive therapy), then inhibition of HIV replication in a patient may permit some immune restoration to occur or, at a minimum, prevent further clinical deterioration, thereby prolonging survival.

Anti-retroviral therapy may also address other disease manifestations of HIV infection. Since the first observation that the brain is an important site of HIV replication, there has been a growing recognition that serious neurologic

dysfunction, ranging from peripheral neuropathy to profound dementia, may be caused by this virus. While it is still not known precisely how HIV enters the nervous system (one likely mechanism is that it is transported there by infected macrophages) or how it induces neurologic damage, it is clear that successful therapeutic strategies must somehow address the consequences of viral replication within the nervous system. As we will discuss later, improvements in the neurologic disease caused by HIV are within the reach of the anti-retroviral chemotherapy regimens now being used within the Clinical Oncology Program.

Stages in the life cycle of HIV that may be targets for anti-viral agents.

Before turning our attention to a discussion of certain research clinical developments in the anti-retroviral therapy of AIDS within the program, it is perhaps worthwhile to review briefly the replicative cycle of HIV and consider how specific stages of viral replication might be targets for therapeutic strategies (Table I). As noted above, HIV belongs to a family of RNA viruses known as retroviruses. By definition, such viruses must replicate through a DNA intermediate (i.e., at one step in their cycle of replication, genetic information flows from RNA to DNA, a "retro" direction). Many members of this family of viruses can induce neoplastic transformation in infected cells, and the terms "RNA tumor virus" or "leukemia virus" were often previously used to describe these viruses. HIV, however, has not been shown to be transforming per se although it is clearly linked to the causation of certain cancers through its ability to induce immunosuppression, and this provides a powerful reminder of the relationship between abnormalities in the immune system and neoplasms. It is also worth stressing that HIV is more complex than previously characterized retroviruses and has at least 8 genes.

The first step in the infection of a target cell by HIV is its binding to the cell surface; in the case of helper-inducer T cells, the binding receptor is believed to be linked to the CD4 antigen. There is recent evidence that this binding between HIV envelope glycoprotein and the CD4 antigen may play a major role in virally-induced T cell lysis through syncytia formation. This binding might possibly be blocked by antibodies either to the viral envelope glycoprotein or to the CD4 antigen; alternatively, synthetic ligands could be used to block viral attachment. During the past year, we have explored certain ligrands, such as peptide T. and those studies will continue in the coming year. There is substantial variation in the envelope of different isolates of HIV, so that antibody directed against one isolate may theoretically not block binding of another. The envelope glycoprotein, however, is constrained by its need to bind to CD4, and it is likely that a relatively invariant region of the envelope will be shown to be crucial for viral infection. During the past year a major search for human monoclonal antibodies against HIV was initiated, and this activity will continue in the coming year.

After attachment, HIV enters a target cell by an incompletely defined mechanism which may involve a fusion process. It then loses its envelope coat, and viral RNA (along with reverse transcriptase) is released into the cytoplasm.

TABLE I. Stages in the replication cycle of a pathogenic human retrovirus which may be targets for therapeutic intervention

Stage	Potential Intervention
Binding to target cell	Antibodies to the virus or cell receptor
Early entry into target cell	Drugs that block fusion or interfere with retroviral uncoating
Transcription of RNA to DNA by	Reverse transriptaste inhibitors
Degradation of viral RNA of an RNA-DNA hybrid	Inhibitors of RNase H activity
Integration of DNA into host genome	Drugs which inhibit pol gene-mediated "integrase" function
Expression of viral genes	"Anti-sense" constructs; inhibitors of tat-III protein or art/trs protein
Budding of virus	Inteferons
Viral component production and	Myristylation, glycosylation and protease inhibitors or modifiers

Compounds which block these steps have been found for other viruses (e.g., amantadine is believed to block the uncoating of influenza A virus, and similar agents may be developed for HIV.

At this point, an HIV pol product, reverse transcriptase, makes a complementary (first strand) strand DNA copy of the RNA genome using a lysine transfer-RNA as the primer This same enzyme then catalyzes the production of a positive strand DNA copy so that eventually, the genetic information is encoded in a double stranded DNA form. Reverse transcriptase activity is essential for viral replication and can for all practical purposes be considered a unique viral function. For retroviruses as a class, it is thought that an RNase H activity is situated at the carboxyl terminus of the reverse transcriptase domain, and this RNase H eliminates the viral genomic RNA strand making it possible for the reverse transcriptase to generate the second DNA strand. If a DNA copy of the uncoated viral RNA is not made promptly, the RNA is susceptible to degradation by cellular enzymes. For these reasons, reverse transcriptase has been a major target for anti-viral therapy in AIDS, and indeed, as will be discussed below, most of the agents now being investigated act at this step of viral replication. It should be noted that the progress in this area has been possible in part because of pioneering research directed at finding agents which inhibit the reverse transcriptase of murine and avian retroviruses.

The DNA copy of retroviral genetic material may become circularized during or soon after its formation and may remain in an unintegrated form or become integrated into the host cell genome through a viral endonuclease or "integrase" (thought to be a pol gene product) which mediates this step. It is possible that drugs which interfere with the "integrase" could be of value. At some later point, the DNA is transcribed to mRNA and to viral genomic RNA using host RNA polymerases, and the mRNA is translated to form viral proteins, again using to a large extent the biochemical apparatus of the host cell. One broad spectrum anti-viral drug, ribavirin, is believed to interfere with a process called 5'-capping of virus-specific mRNA in other viral systems and it may possibly have a related activity in HIV infection. During the past year, we have studied the anti-retroviral activity of ribavirin. In our hands, the drug does not have a good anti-retroviral effect "in vitro". However, it may potentiate certain purine anti-retroviral nucleosides, and the mechanism for this effect in now under study.

It has recently been shown that the production of HIV proteins is under the regulatory control of at least two viral genes, one named tat- III and the other named art or trs. The products of these unique viral genes are absolutely required for efficient HIV replication, and they might be future targets for anti-viral therapy. The product of the tat-III gene, for example, is believed to markedly increase production of viral proteins after binding to a region on what is called the 5'long terminal repeat (LTR) of the viral mRNA. A cluster of positively charged amino acids in the protein encoded by tat-III has been postulated to be the site of its binding to mRNA. Drugs might be developed (perhaps designed on the basis of x-ray diffraction studies and computer-generated models of the protein with the goal of binding to and inhibiting the function of tat- III.

Another approach which may conceivably be used to inhibit the production of viral proteins may be the use of "anti-sense" oligodeoxynucleotides. These are short sequences of DNA (or DNA which has been chemically modified to confer better cellular penetration and resistance to enzymatic degradation) with base pairs that are complimentary to a vital segment of the viral genome. Such oligonucleotides could theoretically block expression of viral proteins through a hybridization arrest of mRNA or by interference with the binding of a regulatory protein (e.g., tat-III) or mnr. In an accompanying report, we discuss a novel class of oligodeoxynucleotides, which are linked by phosphororate containing diesters. Some agents in this novel class of compounds can suppress HIV replication by a process that appears to depend, in part, on such a hybridization arrest.

The final steps of viral replication involve secondary processing of certain viral proteins by proteases (thought to be a function of one of the viral pol gene products and myristylating and glycosylating enzymes (provided by the host cells); these steps could be affected by inhibitors of these enzymatic functions. Finally, the virus is released by budding; alpha interferon, which has been shown to inhibit HIV replication, is thought to possibly act at this step. It has become evident that certain cells, including macrophages, EBV-infected B cells (which are increased in patients with AIDS), and even certain T cells may be chronically infected with HIV without necessarily being destroyed by the virus.

Agents which act at the later steps of viral replication (beyond reverse transcription and integration) may theoretically address the problem of these chronically infected cells, while those agents which act at reverse transcription or earlier steps may be effective at protecting uninfected cells from HIV infection. A combination of early and late acting agents may therefore be more effective than either used alone. Combined therapy in addition may reduce the chance of the virus developing resistance to any single agent.

2',3'-Dideoxynucleoside analogues as inhibitors of HIV infection

We would now like to turn our attention to a group of compounds, 2',3'dideoxynucleoside analogues, which can be metabolized in mammalian cells to become potent inhibitors of HIV replication. 2',3'-dideoxynucleosides have previously been studied in several pioneering laboratories. Our group has observed that some of these compounds block the infection of target cells by HIV at concentrations which are 10 to 20 fold lower than those at which they inhibit the proliferation and survival of the cells, even under conditions of high multiplicity of infection. As will be discussed further below, one drug, the 3'-azido derivative of 2',3'-dideoxythymidine (3'-azido-2',3'-dideoxythymidine, AZT), has recently been shown to confer a survival advantage when administered to certain patients with AIDS. The 2',3'-dideoxynucleosides which have been shown to inhibit HIV replication differ from the normal substrates for nucleic acid synthesis (2'deoxynucleosides) in that the 3'-hydroxy (-OH) group is replaced by hydrogen (-H) or another group which cannot form phosphodiester linkages (Fig. 1). Individual compounds can be metabolized by mammalian kinases to 5'-triphosphate forms, but the degree of anabolic

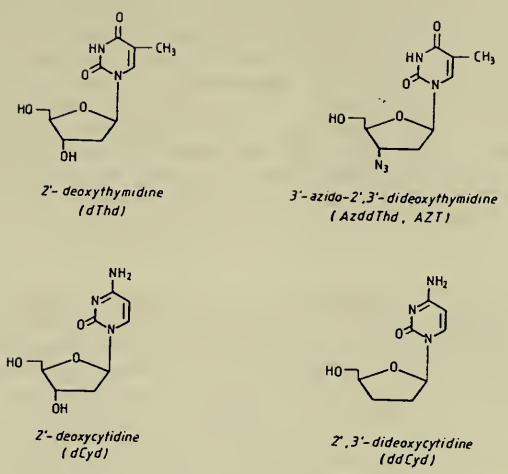

Fig. 1. Chemical structures of two dideoxynucleoside analogues now in clinical trials (right) compared to their respective physiologic nucleoside counterparts (left).

phosphorylation cannot be extrapolated from one drug to another and must be determined on a case by case basis. There is evidence that these phosphorylated nucleosides inhibit HIV replication by competively inhibiting reverse transcriptase or acting as chain terminators; because of the 3' modification, once viral reverse transcriptase is fooled into adding them to the end of a growing chain of DNA, no subsequent 5'>3' phosphodiester linkages can be made (Fig. 2). The DNA polymerase of HIV (reverse transcriptase) and other retroviruses is much more susceptible to the inhibitory effects of these compounds (as triphosphates) than is mammalian DNA polymerase alpha, and this is most likely one basis for their selective anti-retroviral activity. Indeed, one might say based on the very low K_m values observed that reverse transcriptase prefers 2',3'-dideoxynucleotides to normal nucleotides as substrates.

As noted above, 2',3'-dideoxynucleosides must be phosphorylated to an active 5'-triphosphate moiety by host cellular kinases. Different species of animals (or even cell types within one species) differ in their efficiency of phosphorylation of these drugs, and for this reason, extreme caution must be used in extrapolating from experimental results obtained in one species (or a cell type within a species) to another. For example, 2',3'-dideoxythymidine is poorly phosphorylated in human cells (K_i/K_m for thymidine kinase ~130; comparing drug to normal substrate), and is among the least potent of any 2',3'-dideoxynucleoside tested. Substitution of the 3'-hydrogen by an azido (N_3) group in the erythro configuration, however, produces a compound mentioned above, 3'-azido-2',3'-dideoxythymidine (AZT) (Fig. 1), that is an excellent substrate for human thymidine kinase (k_m:3μM; K_i/K_m ~1) and extremely efficient at inhibiting HIV replication in vitro. The threo configuration is very much less potent as an anti-retroviral agent (Mitsuya and Broder, unpublished).

During the past year, we have observed that the 3'-cano analogue of 2',3'-dideoxythymidine is also an excellent inhibitor of HIV replication in vitro.

Of all the nucleoside analogues that have been tested so far in our laboratory for the ability to inhibit the in vitro replication and cytopathic effect of HIV, the pyrimidine analogue 2',3'-dideoxycytidine (ddCyd) is the most potent on a molar basis. At very high multiplicity of infection, essentially complete inhibition is obtained at a concentration of 0.5 μM and the virus-inhibiting effect is durable over prolonged culture periods. At low multiplicity of infection, viral suppression may be seen at concentrations of 10 nanomolar or less in vitro.

During the past year, we studied the biochemical pharmcology of ddCyd. The metabolism of ddCyd to its 5'-mono-,5'-di-, and 5'-triphosphate metabolites is similar in uninfected and HIV-infected ATH8 cells (a normal helper inducer T cell line immortalized after transformation with HTLV-I and highly sensitive to the cytopathic effect of HIV. This observation suggests that no specific viral enzyme is involved in the activation and metabolism of the drug (Fig. 3). The following observations led us to conclude that ddCyd is phosphorylated by the cellular deoxycytidine (dCyd) kinase to its 5'-monophosphate derivative ddCMP:

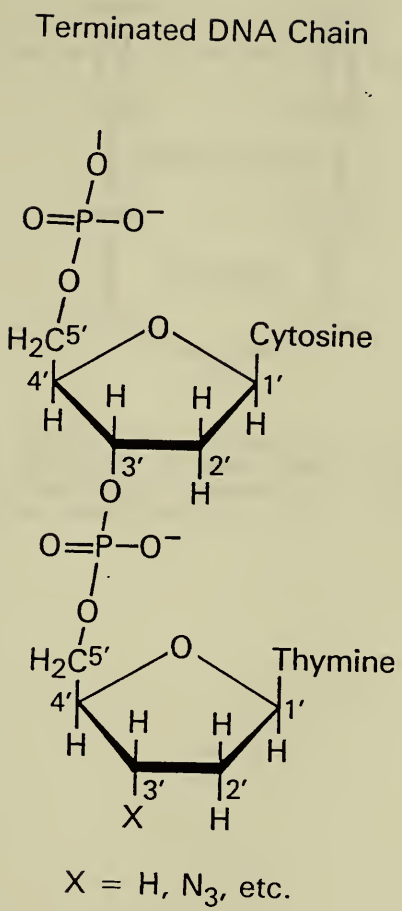

Fig. 2. One mechanism to explain the antiretroviral activity of 2',3'-dideoxynucleoside analogues. After anabolic phosphorylation within a target cell, 2',3'-dideoxynucleotides can be incorporated into a growing chain of DNA as the genomic RNA of HTLV-III is transcribed to DNA; this will elongate the DNA by one residue and then terminate DNA synthesis because the 3'-carbon is not available for further phosphodiester linkage.

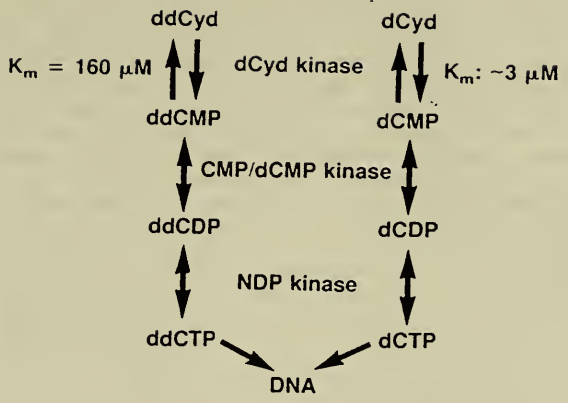

Fig. 3. Pathways of anabolic phosphorylation for 2',3'-dideoxycytidine (ddCyd) and its normal counterpart 2'-deoxycytidine (dCyd). The K_m for the first step of anabolic phoshorylation (mediated by 2'-deoxycytidine kinase) is shown for both drug and the normal substrate.

(i)the cytostatic effects of ddCyd against human Molt/4F cells are dramatically reversed by dCyd (> 150 fold) ; (ii) the anti-HIV effects of ddCyd in ATH8 cells are reversed by dCyd; (iii) dCyd severely depresses intracellular phosphorylation of ddCyd, (iv) ddCyd lacked any appreciable cytostatic effect against a dCyd kinase-deficient cell line (ID_{50}: >2400 uM compared to 320 uM for the wild-type cell line; (v) phosphorylated metabolites of ddCyd could not be detected in a dCyd kinase-deficient cell line which contains normal levels of Cyd/Urd kinase. However, we have found that dCyd kinase has a much lower affinity for ddCyd (K_m ~160 μM) than its physiological substrate dCyd (K_m 3 μM) unpublished). Since ddCTP is thought to be the active intracellular species for the antiretroviral effect of ddCyd, limited intracellular levels of ddCTP or high intracellular ratios of dCTP versus ddCTP could prevent ddCyd from achieving its optimal antiviral effect. Nevertheless, we could demonstrate that ATH8 cells as well as a number of other human cell lines including PHA stimulated normal lymphocytes developed intracellular ddCTP levels of about 0.5 uM when incubated with 1 μM ddCyd for 24 hours. These ddCTP levels exceed the Ki-value of ddCTP for HIV reverse transcriptase 0.2 uM) determined by Hao Z, Johns DG, Broder S, et al., unpublished. Moreover, Mitsuya et al showed that ddCTP could bring about a chain termination of DNA synthesis catalyzed by HIV reverse transcriptase at a concentration which is 40 fold below the dCTP concentration present in the reaction mixture. Taking into account that the levels of ddCTP attainable upon incubation of human lymphocytes with 1 μM ddCyd are≥ 1/40 the normal intracellular dCTP levels present in the cells [e.g., 20 μM in human H9 cells, we may conclude that these ddCTP levels are high enough to account for an antiretroviral (chain-terminating) effect of ddCyd. However, preliminary studies during the past year suggest that the biochemical pharmacology of this drug (and other drugs such as AZT) might be different in machrophages.

We are exploring ways to manipulate the activity of certain mammalian enzymes involved in pyrimidine nucleoside metabolism, in particular dCyd kinase and CDP reductase, in order to increase the anabolic phosphorylation of this drug. One potential approach is to decrease the intracellular dCTP pools. Since dCTP is a potent feedback inhibitor of dCK a reduction of the dCTP levels will stimulate dCyd phosphorylation and at the same time enhance ddCyd phosphorylation.

In Fig. 4, potential target enzymes for achieving decreased dCTP pools are indicated. All inhibitors shown in Fig. 4, have previously been demonstrated to decrease dCTP pools. We found that a 12-hour pre-incubation period of murine leukemia L1210 cells with hydroxyurea [an inhibitor of CDP reductase], 3-deazauridine (an inhibitor of CTP synthetase, and dThd [an allosteric inhibitor of CDP reductase as its 5'triphosphate derivative as well as an activator of dCyd kinase under certain conditions], led to a 4-6 fold increase in phosphorylated ddCyd metabolites. Pyrazofurin (an inhibitor of OMP decarboxylase increased ddCTP levels only 2-3 fold while PALA [an inhibitor of aspartase transcarbamoylase] showed only marginal stimulation under our experimental conditions. A 4-quinoline carboxylic acid derivative, an inhibitor of dihydroorotate dehydrogenase and 6-azauridine, an inhibitor of OMP decarboxylase, were without any effect. However, it should be emphasized that optimal exposure time and concentration of these antimetabolites remain to be determined for each particular compound.

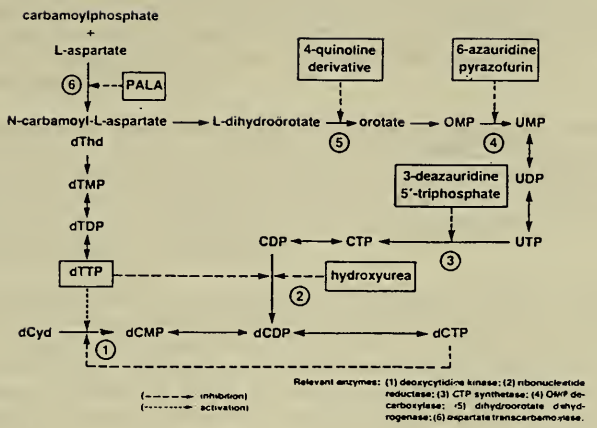

Fig. 4. Potential target enzymes for achieving a decreased intracellular concentration of dCTP.

We then focused on dThd in more detail. When the effect of dThd on the stimulation of ddCyd phosphorylation was examined more closely, we found that the degree of stimulation of ddCyd phosphorylation was highly dependent on preincubation time as well as concentration of dThd. We observed a correlation between the increased ddCTP levels and decreased dCTP pools. However, we also found that the drop in dCTP pools seemed necessary but probably not sufficient to obtain efficiently increased ddCTP levels.

Finally, we demonstrated that under specified conditions, dThd enhanced the protective effect of ddCyd in HIV exposed ATH8 cells. Moreover, the dThd-induced increase in the concentration of phosphorylated ddCyd metabolites did not parallel the slight increase in apparent toxicity. Therefore, toxic and antiviral effects may be modulated by different mechanisms, an observation of potential chemotherapeutic importance.

The possible mechanisms for the stimulation of ddCyd phosphorylation are as follows (Fig. 5): (i) Conversion of dThd to dTTP in the cells, the latter metabolite being capable of enhancing the activity of ddCyd kinase under constant dCTP levels (ii) inhibition of CDP reductase by dTTP which would result in decreased amounts of dCTP, a potent feedback inhibitor of dCyd kinase, consequently, dCyd kinase will be stimulated and thus enhance ddCyd phosphorylation. (iii) The drop in dCTP pools will increase the competition of ddCTP with dCTP for reverse transcriptase, the assumed target enzyme for the antiviral effects of ddCyd. Thus, a combination of ddCyd with dThd should potentially be able to lead to increased intracellular ddCTP pools. Moreover, dThd has already been used in phase I clinical trials and should be a good candidate to be included in treatment schedules for AIDS patients in the near future. Indeed, as will be discussed later, the toxic effect of AZT is probably caused by a severe drop in dTTP levels; therefore, a schedule for drug administration in AIDS patients may be considered in which several weeks treatment with high concentrations of AZT is followed by several weeks treatment of a combination of ddCyd with dThd. This treatment regimen may result in an increased chemotherapeutic index by enhancing the antiviral effect and/or lowering (or rescuing) toxicity.

Additional testing has demonstrated that 2',3'-dideoxycytidine has other properties desirable in a potential therapeutic agent for HIV infection: it is relatively resistant to cytidine deaminase (a major catabolic enzyme for cytidine analogues) it is well absorbed when administered by the oral route; it has straightforward pharmacokinetic clearance by the kidney; it does not appear to induce pyrimidine starvation; and finally, it has comparatively little toxicity in test animals. Entry to the central nervous system in monkeys is also rapid, but the cerebrospinal fluid to plasma concentration ratio after i.v. administration is not as high as AZT. We have initiated a Phase I (feasibility and toxicity) study of 2',3'-dideoxycytidine in patients with AIDS as the next step in the development of this drug. A comparable study for a purine analogue, 2',3'-dideoxyadenosine, is planned for the future. The preliminary data using dideoxycytidine in patients are summarized below.

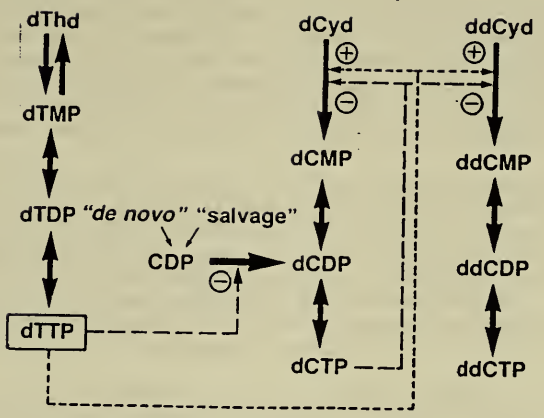

Fig. 5. Possible mechanism for stimulating ddCyd phosphorylation and reducing dCTP formation using thymidine (dThd). The + sign indicates catalytic steps that can be enhanced. The - sign indicates catalytic steps that can be inhibited.

During the past year, appropximately twenty patients have been enrolled in an escalating dose Phase I study of ddCyd. The drug exhibited a straightforward pattern of doseindependent renal clearance. At doses of .03 mg/kg/q4h or higher, it was possible to exceed the virustatic levels predicted from our earlier in vitro work. The drug has an oral bioavailability that exceed 70% in patients, and we found an unexpected high relative capacity to cross the blood brain barrier. The cerebrospinal fluid concentrations (at 3-hours) ranged from 9% to 37%, with a mean value of approximately 20%.

The profile of toxicity was interesting. Some patients developed a syndrome of non-exfoliative rash and apthous ulcers usually beginning on the tenth day of the study. These side-effects generally cleared with continued administration of the drug. The drug does not appear to cause the megaloblastic anemias that are the hallmark of AZT, and anemia was rarely a problem. At higher doses, some patients developed transicent leukopenia and thrombocytopenia. Some patients after the third or fourth month of continous therapy developed what appears to be a reversible small fiber peripheral neuropathy.

Although a Phase I study does not provide a proper setting to draw conclusions about efficacy, ddCyd did appear to improve certain antigenspecific assays if in vitro immune reactivity in patients. Moreover, some patients exhibited a dose-dependent reduction in their circulating levels of HIV p24 antigenemia, a measure of in vitro viral replication, which from other studies appears to correlate with the overall course of the disease.

Another dideoxynucleoside analogue already touched upon, 3'-azido-2',-3'dideoxythymidine (AZT), was synthesized >20 years ago by Horwitz et al. and was shown to inhibit C-type murine retrovirus replication in vitro by Ostertag > 12 years ago. In the beginning of 1985, our group, in collaboration with the Wellcome Research Laboratories, observed that AZT at concentrations of 1 to 3 μM inhibited HIV replication even under test conditions that utilize a high multiplicity of infection. (Under less stringent test conditions, substantially lower concentrations of AZT can be shown to be inhibitory).

AZT metabolism to its 5'-triphosphate metabolites is similar in uninfected and HIV infected H9 cells indicating that no specific viral enzyme is involved in the activation and metabolism of the drug (Fig. 6). AZT is phosphorylated by the cellular dThd kinase (TK) to AZT-5'-monophosphate (AZT-MP). Its Km for this enzyme nearly equals that of the physiological substrate dThd (2 μM); the Ki/Km ratio of AZT for dThd kinase (as measured with radiolabeled dThd as the substrate) is close to 1, inhibition of TK is competitive with respect to dThd, and its V_{max} is 60% of the V_{max} for dTMP formation These data suggest that AZT is an excellent substrate for TK to be converted to its 5'-monophosphate derivative.

When evaluated for its substrate properties for thymidylate kinase (dTMP-K), AZT-MP shows a Km of 8 μM, that is a 2-fold higher value than the Km for the physiological substrate dTMP (Fig. 6). However, V_{max} is only 0.3% of V_{max} for dTMP formation. Thus, AZT-MP has to be considered as a potent substrate inhibitor of dTMP kinase. Preliminary observation suggest that the 3'-cyano-congener discussed earlier has less of a capacity to inhibit thymidine kinase.

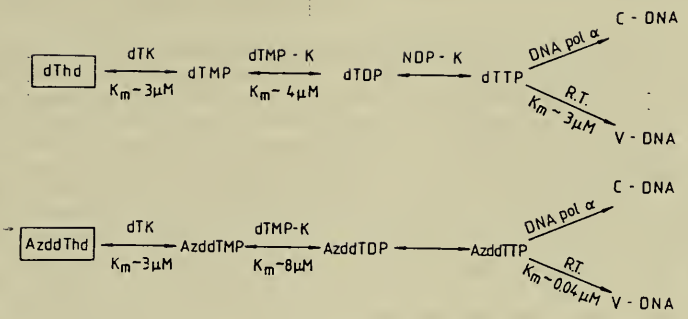

Fig. 6. Anabolic phosphorylation of thymidine (dThd) and the 3'-azido analogue of 2',3'-dideoxythymidine (AzddThd). The final normal nucleotide product (dTTP) and drug product (AzddTTP) can be incorporated into both cellular DNA (cDNA) or retroviral DNA (vDNA). However, the viral DNA polymerase (R.T.) has a high affinity for AzddTTP, and this at least partially accounts for the selectivity of AzddThd as an antiretroviral agent. Also, see Fig. 2. Some of the toxicity of AzddThd appears to be related to the reduction of dTTP caused by the drug in inhibiting thymidylate kinase (dTMP-K) (see text).

Although the substrate properties of AZT-DP for nucleotide5'-diphosphate kinase (NDP-K) have not yet been determined or reported, one may consider dTMP-K as the most likely rate-limiting step in the conversion of AZT to its active metabolite AZT-5'-triphosphate (AZT-TP).

Once converted its 5'-triphosphate derivative, AZT may theoretically interfere with all cellular DNA polymerases (i.e., DNA polymerase α, β, γ) and the viral DNA polymerase (reverse transcriptase). The observation that HIV reverse transcriptase has a very high affinity for AZT-TP (Km=.04 μM), and this affinity is several orders of magnitude higher than the affinity for cellular DNA polymerase α and β, Figure 6), may explain the selectivity and potency of AZT as an anti-retroviral chemotherapeutic agent in human beings. Thus, assuming that inhibition of reverse transcriptase is the main target for the observed antiviral effects of AZT, low intracellular ratios of dTTP versus AZT-TP will increase the potency of AZT as an antiretroviral agent, but may also enhance the cytostatic and/or cell toxic effects of the drug. Therefore, determination of intracellular nucleotide pool levels are necessary to obtain more information concerning intracellular balances between phosphorylated AZT metabolites and the natural nucleotide pools and to give a rationale in our attempt to manipulate the antiviral and/or action of the drug.

When H9 cells are incubated with AZT, dramatic changes in the dTTP pools occur: they drop at least 20-fold. How can the change of the dTTP pools be explained and what is their relevance in the toxic effects of AZT? Most likely, the decrease of intracellular dTTP levels is due to the inhibition of dTMP-K by AZT-MP. Since every flow of dThd to dTTP either formed by salvage or de novo synthesis, has to go through this enzyme, it is obvious that a block at this level results in a decrease of dTDP and dTTP pools (consumed during the remaining DNA synthesis and/or temporary accumulation of dTMP. Indeed, we observed increased dTMP levels and decreased dTTP levels in AZT-treated ATH8 cells. Thus, starvation of dTTP by AZT treatment will shut down DNA synthesis and may lead to the equivalent of a thymineless death of the cells. However, as a consequence of the inhibition of dTMP-K by AZT-MP, a dramatic accumulation of AZT-MP will also occur. This phenomenon has been demonstrated by Furman et al in H9 cells and by Balzarini and co-workers in human ATH8 and Molt/4F cells and in caprine TAHR cells (unpublished). Under some experimental conditions, AZT-MP pools exceed AZT-TP pools by 50-100 fold and intracellular levels of AZT-MP as high as 800 uM could be recorded in H9 cells treated with 50 μM AZT. The extensive accumulation of AZT-MP into cells makes this metabolite also a potential candidate for thymidylate synthetase (TS) inhibition. Indeed, TS represents a key enzyme in DNA synthesis since it is the only de novo source for the cells to synthesize dTMP, and it has been demonstrated that inhibition of this enzyme may lead to potent inhibition of cell growth and DNA synthesis. However, when AZT-MP is evaluated for its inhibitory effect against partially purified TS from Molt/4F cells, no significant inhibition could be demonstrated at an AZT-MP concentration as high as 250 μM. Also when evaluated in intact cells by measuring the effect of AZT on tritium release from [5-^{3}H]dUrd and [5^{3}H]dCyd, no inhibition by 10 uM of AZT was recorded even when tritium release was measured at different time points (up to 72 hours) during AZT incubation. Deoxycytidylate (dCMP) deaminase, another important source of the substrate dUMP for the thymidylate synthetase reaction and thus a

potential target for toxicity, is also not affected by AZT and its phosphorylated metabolites. Thus, it is unlikely that inhibition of TS or dCMP deaminase by AZT or its phosphorylated metabolites accounts for the observed drop in dTTP pools. Morever, since addition of dThd could easily overcome the inhibitory effect of AZT against Molt/4F cell proliferation, it seems likely that the severe decrease in dTTP levels and the toxic effects achieved by AZT must be ascribed to an inhibition of the dThd flow to dTTP at the dTMP-K level by AZTMP. A similar observation has been observed by us for 2',3'-ddThd which also accumulates as its 5'-monophosphate metabolite upon administration to Molt/4F and ATH8 cells although phosphorylation occurs considerably less extensively than for AZT. These data suggest that the phenomenon of inhibition of dTMP-K by AZT-MP and ddTMP may be a general property of 2',3'-dideoxythymidine analogues and one may speculate that toxicity exerted by this subset of compounds will be primarily due to dTTP starvation in human target cells.

However, inhibition of dTMP-K by AZT-MP is not a general phenomenon when cells from different species are compared. Indeed, we found dramatic differences in metabolism of AZT in murine leukemia (L1210), caprine ovary (TAHR) and human lymphoblast (Molt/4F) and lymphocyte (ATH8) cells. While accumulation of AZT-MP was noted in the human lymphoid and caprine ovary cells (ratio AZT-MP/AZT-TP:≈50-200), extensive formation of AZT-TP versus AZT-MP was noted in murine leukemia L1210 cells (ratio AZT-MP/AZT-TP: 0.5) (Fig 7). These data clearly indicate that metabolism of AZT is highly dependent on the species and cell type. This may also explain the observation that AZT is a potent inhibitor of mouse viremia and retroviral disease (e.g., splenomegaly) in Rausher murine leukemia virus infected mice. It also explains our observation that in Moloney murine sarcoma virus infected newborn NMRI mice, AZT treatment severely depresses tumor formation and dramatically prolongs survival rate of the test mice. These protective effects against murine retroviruses are probably due to the extremely high levels of AZT-TP found in murine cells (versus human cells), rather than the species origin of the virus because Dahlberg, Aaronson, Mitsuya and Broder found that AZT dramatically inhibits the replication of an amphotropic murine leukemia virus in cells of the mouse and rat but is only moderately effective in human cells. Thus, one should be warned not to automatically extrapolate metabolic data found in murine or other animals cell lines, to human cell metabolism.

Evaluation of azidothymidine (AZT) in patients with advanced HIV-diseases

In the middle of 1985, we began administering AZT to patients with AIDS and related conditions in a phase I trial conducted in collaboration with Duke University Medical Center and Wellcome Research Laboratories. One of the purposes of this trial was to study the pharmacokinetics of AZT. We found that the drug has good oral bioavailability (approximately 60%) and that levels of AZT that were inhibitory for HIV replication in vitro were achievable in patients. The half-life of AZT was found to be approximately 1 hour with much of the drug clearance being via

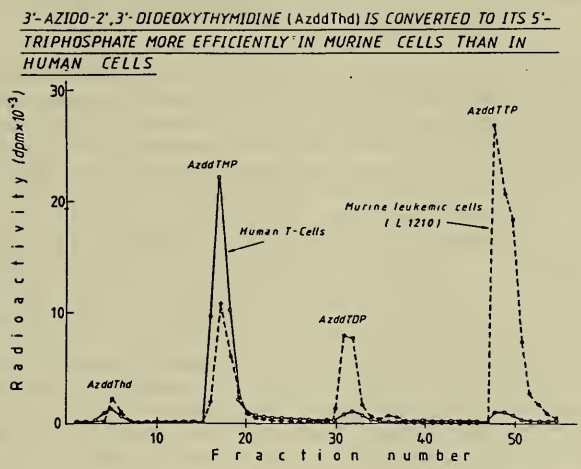

Fig. 7. Different profiles of anabolic phosphorylation of AzddThd in human T cells versus mouse L1210 cells. The antiretroviral effects and possibly some toxic effects of the drug are mediated by the 5'-triphosphate derivative (AzddTTP). Note that in human cells, only a small fraction of the drug is metabolized to a triphosphate form.

glucuronidation. (At least one drug which is known to interfere with hepatic glucuronidation, probenecid, increases the half life of AZT [102], and it is possible that other inhibitors of glucuronidation such as acetaminophen, morphine, and sulfonamides could have similar effects.) Finally, AZT was found to have excellent penetration across the blood-brain barrier.

In this trial and extensions of this trial, we also observed that patients administered intermediate doses of AZT (15 to 30 mg/kg/day orally) over a 6 week period have partial restoration of immune function including 2 to 3 fold increases in the absolute number of helper-inducer (CD4+) T cells and the development of cutaneous delayed type hypersensitivity reactions in previously anergic individuals. These changes often are detectable after the second week of therapy, but they are not always dramatic. Indeed, one of the principles we now know is that small increments of T4 counts can confer a significant survival advantage to patients. The majority of patients given AZT also gain weight, report less fatigue, and have improvement in other clinical abnormalities associated with AIDS. Finally, we have observed that some patients with HIV induced neurologic disease (particularly those with dementia) have substantial improvements in neurologic function upon being given AZT. This includes improvements in relative glucose oxidation in the brain as assessed by positron-emission tomography. AZT, however, would not a priori be expected to affect secondary neurological manifestations of AIDS such as toxoplasmosis or central nervous system lymphoma, and these conditions should be excluded in evaluating the effects of AZT on HIV-induced neurologic disease.

Bone marrow toxicity (particularly anemia and leukopenia, and at times including declines in the number of CD4+ lymphocytes) is observed in some patients receiving high doses of AZT (90 mg/kg/day orally) for 4 to 6 weeks and in some patients receiving lower doses of AZT (25 to 30 mg/kg/day orally) for more than 8 weeks. Increase in the red blood cell mean corpuscular volume (reflecting megaloblastic changes) is often an early sign of this toxicity. Interestingly, thrombocytopenia is much less common and indeed, patients often have increases in the number of platelets upon being given short courses of AZT Some patients receiving AZT report mild headaches, and two patients receiving high doses (90 mg/kg/day orally) developed transient agitation. In summary, bone marrow suppression appears to be the major limiting toxicity of this drug.

We have now administered AZT to certain patients with AIDS for as long as 24 months, and some patients appear to tolerate the drug over this period of time, although patients with fulminant AIDS often required dose reductions. Formal proof that AZT could prolong survival, at least in AIDS patients who had recently recovered from Pneumocystis carinii pneumonia, was provided by a multicenter doubleblind placebo-controlled trial in which there was a dramatic difference in mortality between the treated and untreated patients.

The trial was begun in February 1986. By the end of September 1986, there were 19 deaths in the placebo arm and one death in the AZT arm. At the same time, patients in the drug arm showed other evidence of clinical

and immunological improvements. (Interestingly, while patients in the AZT arm, in addition to an improved survival, had a clear reduction in the number of opportunistic infections or other AIDS-related events, a major difference in this regard between the two arms of the study did not occur until after six weeks into the study, suggesting that the benefits conferred by AZT did not fully materialize until at least six weeks of administration.) At that point, an independent data-monitoring board recommended that the placebo group begin to receive the drug. The study is still underway (with both arms now receiving drug to determine long-term safety and efficacy.

AZT is now available to physicians for the treatment of patients with AIDS who have had Pneumocystis carinii pneumonia and who fit certain other criteria as a prescription drug. It is worth stressing that the clinical experience with AZT is at this time limited, and the availability of AZT represents only a first step. The optimal dose for long term therapy is still being explored. During the past year, we have also initiated a pilot study to see if a regimen of AZT alternating on a weekly basis with ddCyd will show superior clinical activity than either agent alone. The non-overlapping toxicity of these drugs provides us with a cautious optimism for such a study. We also need to learn the best schedule of administration, and to learn whether the development of AIDS may be slowed or prevented by administering AZT early in the course of HIV infection. We are just beginning to explore strategies which may permit a greater antiviral activity without a concomitant increase in bone marrow toxicity. In this regard, our group has recently observed that acyclovir (a guanosine analogue with potent activity against Herpes viruses but little or no anti-retroviral activity per se) can potentiate the anti-retroviral effect of AZT in vitro through an as yet unknown mechanism Acyclovir does not characteristically cause bone marrow suppression and it is possible that the combination of these two agents may be of benefit. We are presently initiating a clinical trial to test this hypothesis. During the past year, we have also initiated a pilot study to see if a regimen of AZT alternating on a weekly basis with ddCyd will show superior clinical activity than either agent alone. The non-overlapping toxicity of these drugs provides us with a cautious optimism for such a study.

Thus, AZT is a drug selected on the basis of its in vitro anti-viral effect against HIV and shown to confer at least a short-term clinical benefit in patients with advanced disease. In this respect, it represents a first step in developing practical chemotherapy against pathogenic human retroviruses. While the clinical results may be important in their own right, they are also important because they appear to validate the some of the strategies of therapy discussed here, and they reinforce the idea that therapy for established retroviral infections will eventually be an attainable goal. Within the intramural program, we beleive the data at hand justify a real sense of optimism about the success of future therapies.

CONCLUSION

AIDS and its related disorders are caused by the third known pathogenic human retrovirus (HIV), which infects and destroys helper/inducer T cells, thereby irreparably damaging the immune system. The genome of this virus contains the standard replicative genes found in other retroviruses; however, it

contains several additional genes not previously known. The product(s) of each gene represents a potential target of opportunity in developing new experimental therapies for diseases caused by this virus, and we have discussed several issues related to the viral life-cycle which may be relevant for future therapeutic strategies. We have also discussed some basic pharmacologic princples for designing anti-retroviral treatment. Curative therapy for diseases caused by pathogenic retroviruses will probably not be possible until the molecular biology of the virus and the structural chemistry of key viral products are defined through further basic research. Nevertheless, HIV has revealed enough of its life-history for us to initiate new therapeutic strategies, and it is not necessary to await new breakthroughs before implementing some strategies that we already know have clinical applications in patients with various stages of HIV infection.

It is thus hoped that further research may permit us to more effectively use AZT in these patients or may reveal other agents which are even more effective. Even at this point, however, the demonstration that one dideoxynucleoside analogue has clinical efficacy in some patients with AIDS has removed some of the uncertainty about the rationale for an anti-retroviral intervention in established AIDS and might help to dispel the prevailing sense of hopelessness about this disease.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CM 07207-01 CO

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phosphorothioate analogs of oligodeoxynucleotides

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

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Molecular Pharmacology Laboratory, DCD, FDS
Laboratory of Tumor Cell Biology, NCI

LAB/BRANCH
Clinical Oncology Program, Office of the Associate Director

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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.)*

Nuclease-resistant phosphorothioate analogs of certain oligodeoxynucleotides have been tested in vitro as antiviral agents against human immunodeficiency virus(HIV) in human T-cells. Phosphorothioate analogs complementary to HIV sequences, as well as non-complementary analogs including homo-oligomers, exhibited potent antiviral activity. The antiviral activity was related to the base composition of the analogs, and longer phosphorothioates were more effective than shorter ones. A 28-mer phosphorothioate oligodeoxycytidine (S-dC_{28}) at a concentration of 1uM exhibited potent antiviral activity and inhibited de novo viral DNA synthesis as shown by Southern blot analysis. However, S-dC_{28} failed to inhibit gag expression in chronically infected T-cells assessed by immunofluorescent assay at concentrations up to 25uM. An N^3-methylthymidine-containing phosphorothioate analog, which does not hybridize efficiently in vitro to complementary normal DNA, showed no antiviral activity. A 14-mer phosphorothioate oligodeoxycytidine (S-dC_{14}) synergistically enhanced the antiviral activity of 2',3'-dideoxyadenosine, an anti-HIV nucleoside. Therefore, phosphorothioate analogs of oligodeoxynucleotides could represent a novel class of experimental therapeutic agents against the acquired immunodeficiency syndrome (AIDS) and related diseases. However, their mechanism of action is likely to be complex.

Introduction

Oligodeoxynucleotides (ODNs; Fig. 1A), which are complementary to certain gene messages or viral sequences, referred to as "anti-sense" compounds, have been reported to have inhibitory effects against Rous sarcoma virus and human T-cell lymphotropic virus type III (HTLV-III), now called HIV. However, the susceptibility of the phosphodiester linkage in normal ODNs (n-ODNs; Fig. 1A, I) to degradation by nucleases would be expected to reduce their potency and in vivo persistance as antiviral agents. Methylphosphonate-ODN analogs (M-ODNs; Fig. 1A, II) are resistant to nucleases, and being uncharged, have increased hydrophobicity, which reportedly confers increased cell membrane permeability upon these compounds. M-ODNs have been found to exhibit antiviral activity, but may require high concentrations (typically 100-300uM) in order to elicit strong antiviral effects. Phosphorothioate-ODNs (S-ODNs), in which one of the non-bridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulfur atom (Fig. 1A, III), have several properties that make them potentially advantageous anti-retroviral analogs. They are stable to cleavage by nucleases, and having the same number of charges as n-ODNs, have good aqueous solubility. They also exhibit more efficient hybridization with a complementary DNA sequence than the corresponding methylphosphonate analogs. These factors led us to test certain S-ODNs as antiviral agents against HIV.

Materials and Methods

Synthesis and Purification of Oligodeoxynucleotides and Their Methylphosphonate and Phosphorothioate Analogs.

The n-ODNs, M-ODN, and S-ODNs were synthesized by either the standard procedure or by modification of the previously reported procedure using an automated synthesizer (Applied Biosystems Inc., Model 380-B) by the phosphoroamidite method. Purification was performed by reverse phase high performance liquid chromatography. The details of synthesis and purification of M-ODNs and S-ODNs will be described elsewhere. The presence of P-S bonds in the phosphorothioates was shown using ^{31}P NMR spectroscopy. N^3-methylthymidine was prepared according to the previously published procedure, then converted to the protected phosphoroamidite form and incorporated into the oligomer synthesis.

Sequences of ODNs Tested

The sequences of ODNs tested (Fig. 1B) were chosen for the following reasons; ODN-1,-3 are complementary (anti-sense) sequences to regions of art/trs genes of HIV (HTLV-III BH10 clone) which are essential for viral replication. ODN-2 is the "sense" counterpart of ODN-1. ODN-4 is a "random" sequence that has the same base composition as ODN-1, but has less than 70% homology with any sequence in HTLV-III BH10 clone either as a sense or anti-sense sequence. The factors of base composition and chain length were evaluated with homo-oligomers of dA, dC, and dT in three lengths (5,14, and 28-mer). An N^3-methyl thymine-containing analog of S-ODN-1 (N-Me-S-ODN-1) was used to assess the role of hybridization in the antiviral effect, since N^3-methyl-substitution on thymidine is known to significantly reduce duplex stability. In fact

Fig. 1A

Structure of Oligodeoxynucleotides

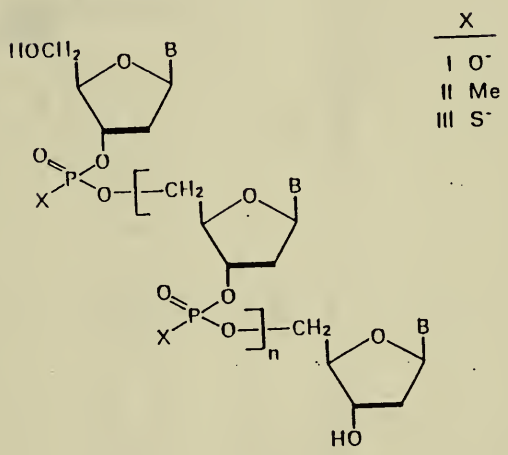
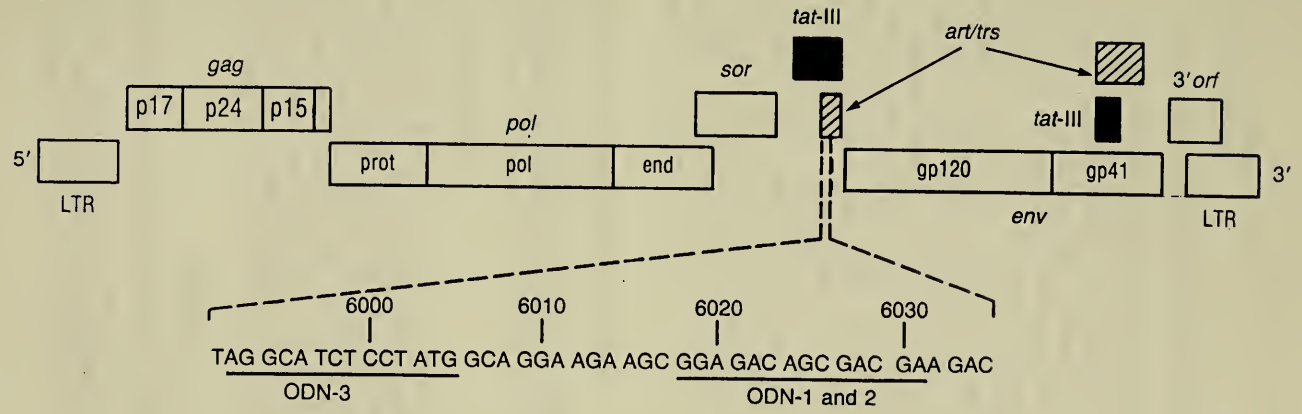


Fig. 1B



Anti-sense: ODN-1: 5'-TCG TCG CTG TCT CC-3'

N-Me-ODN-1: 5'-TCG T*CG CTG T*CT CC-3'

(* denotes N^3-methyl-thymidine)

ODN-3: 5'-CAT AGG AGA TGC CT-3'

Sense: ODN-2: 5'-GGA GAC AGC GAC GA-3'

Random: ODN-4: 5'-CTG GTT CGT CTC CC-3'

dCn: 5'-$(dC)_n$-3'

dAn: 5'-$(dA)_n$-3'

N-Me-S-ODN-1 gave no measurable Tm with its complemetary DNA sequence under physiological conditions.

Cytopathic Effect Inhibition Assay for an Assessment of Anti-HIV Activity.

We previously reported an *in vitro* HIV cytopathic effect inhibition assay for assessing anti-HIV activity. In this *in vitro* assay system, immortalized $T4^+$ T-cells (ATH8 cells) were used as target cells because of their profound sensitivity to the cytopathic effect of HIV. The target cells ($2x10^5$ ATH8 cells) in each tube were pretreated with the stated concentration of each oligomer for 16 hrs, although it was subsequently found this pretreatment was unnecessary, and then incubated with polybrene for 45 min. Following the centrifugation, each set of pelleted cells was incubated with $HTLV\text{-}III_B$ for 1 hr (500 virus particles per cell unless otherwise indicated; this dose is approximately 100 to 1000 times higher than the minimum cytopathic dose in our assay system. Complete media (2ml RPMI 1640) supplemented with L-glutamine (4mM), 2-mercaptoethanol ($5x10^{-5}$M), penicillin (50 unit/ml), and streptomycin (50 ug/ml), and containing 15% fetal calf serum and IL-2 (15% of conventional IL-2 from Advanced Biotechnologies, Inc., Silver Spring, MD, plus 20 unit/ml of recombinant IL-2 from Amgen Biologicals, Thousand Oaks, CA) was used with various concentrations of oligomers added. The number of viable cells were counted in a hemocytometer using the trypan blue exclusion method on day 7 following exposure to the virus. Each set of data in Tables and Figures were obtained from simultaneously performed experiment to make a precise comparison among agents tested.

Determination of HIV gag Protein Expression.

The percentage of the cells expressing p24 *gag* protein of HIV was determined by indirect immunofluorescence microscopy as described by using anti-HIV p24 murine monoclonal antibody (M26; kindly provided by F.D.Veronese and R.C.Gallo).

Southern Blot Analysis.

Target cells($1x10^7$ ATH8 cells) were pretreated with or without S-dC_{28} at various concentrations for 16 hrs, then treated with polybrene, exposed to $HTLV\text{-}III_B$ (500 virus particles per cell), resuspended and cultured in the presence or absence of S-dC_{28}. On days 4 and 7 following the exposure to the virus, high molecular weight DNA was extracted, digested with Asp718 (a Kpn I isoschizomer) (Boehringer-Mannheim, Indianapolis,Ind.), and subjected to Southern blot analysis hybridized with a labelled insert of molecular clone of the *env* region of HTLV-III(BH10) containing a 1.3Kb Bgl II fragment.

Results

Antiviral Activity of Phosphorothioate Analogs.

Results of the antiviral effect and cytotoxicity of ODNs are presented in Table 1. The two n-ODNs and one M-ODN tested showed no significant inhibitory effects, while all the S-ODNs exhibited significant inhibition of

Table 1

COMPOUND	ANTI-VIRAL EFFECT(%)[a]				CYTOTOXICITY(%)[b]			
	1	5	10	25 (uM)	1	5	10	25 (uM)
S-ODN-1[c]	0	43	72	95	0	0	0	20
n-ODN-1[d]	3	2	9	4	35	22	27	14
M-ODN-1[e]	8	20	13	10	20	27	20	20
S-ODN-2	11	56	100	78	0	0	0	6
n-ODN-2	11	9	0	11	18	28	35	32
S-ODN-3	0	6	44	94	0	0	0	13
S-ODN-4	0	53	78	100	0	0	0	0
S-dC_{14}	25	100	100	100	0	0	0	0

the cytopathic effect of HIV. Surprisingly, the 14-mer phosphorothioate homo-oligomer of dC(S-dC_{14}) was found to be the most potent antiviral compound among those tested in this series of experiments. Since phosphorothioate ODNs which are not anti-sense sequences appear to be very effective antiviral agents, we tried to clarify the nature of the base composition effect. Comparing the effects of 5uM of each of the 14-mer phosphorothioates tested, it was found that inhibition of the viral cytopathic effect was approximately linear with respect to the G+C content of the analog (data from Table 1).

Comparison of Anti-HIV Activity in Various Lengths of Oligo-dC and Oligo-dA Phosphorothioates.

Because it is possible that inter-assay variation may create an inappropriate comparison of antiviral activity among agents, we preformed experiment simultaneously to make more precise comparisons.

As illustrated in Fig. 2A, the inhibitory effects of S-dC_n are greater and more persistent than those of S-dA_n for both 14-mer and 28-mer, while 5-mers belonging to both categories fail to significantly inhibit the cytopathic effect of the virus. The order of effectiveness of the homooligomers was dC>dT>dA for 14mer (data not shown for dT). We then explored the effect of oligomer length in detail. The longer sequences were found to be more effective than the shorter sequences at the same molar concentration of nucleotide unit. For example, in Fig.2B the 28-mer S-dC_{28} at concentrations as low as 0.5uM(13.5uM of nucleotide equivalents) gave complete protection against the virus, while the corresponding 14-mer at 5uM (65uM equivalents) had only a moderate effect. The S-dC_{28} gave the most consistent and durable antiviral effects under the conditions used in these experiments. These data suggest a real length effect and argue against either metal ion chelation or degradation to reactive monomers.

Effect of N^3-methyl-thymidine Substitution in ODN Analog on Anti-HIV Activity.

N^3-methyl-thymidine-containing S-ODN-1 showed no anti-HIV activity, while S-ODN-1 consistently exhibited substantial activity against HIV (Fig.3). Since N^3-methyl substitution on the pyrimidine base is known to profoundly reduce hydrogen bonding to complementary adenosine residues, the relative inactivity of this N^3-methyl-thymine-containing analog of phosphorothioate suggests that antiviral activity could be brought about by binding to nucleotide sequences as at least one mechanism.

Inhibition of de novo HIV DNA Synthesis in ATH8 Cells Exposed to the Virus by 28-mer of Oligodeoxycytidine Phosphorothioate.

Fig. 4 shows the inhibitory effect of the phosphorothioate oligodeoxycytidine analog (S-dC_{28}) on de novo HIV DNA synthesis in target cells. On days 4 and 7 following the exposure to the virus, a substantial amount of viral DNA was detected by Southern blot analysis without antiviral agents. S-dC_{28}, as well as 2',3'-dideoxyadenosine as the positive control, significantly inhibited the de novo synthesis of viral DNA at concentrations as low as 1uM.

Failure to Inhibit the Expression of Viral Protein by 28-mer Oligodeoxycytidine

Fig. 2A

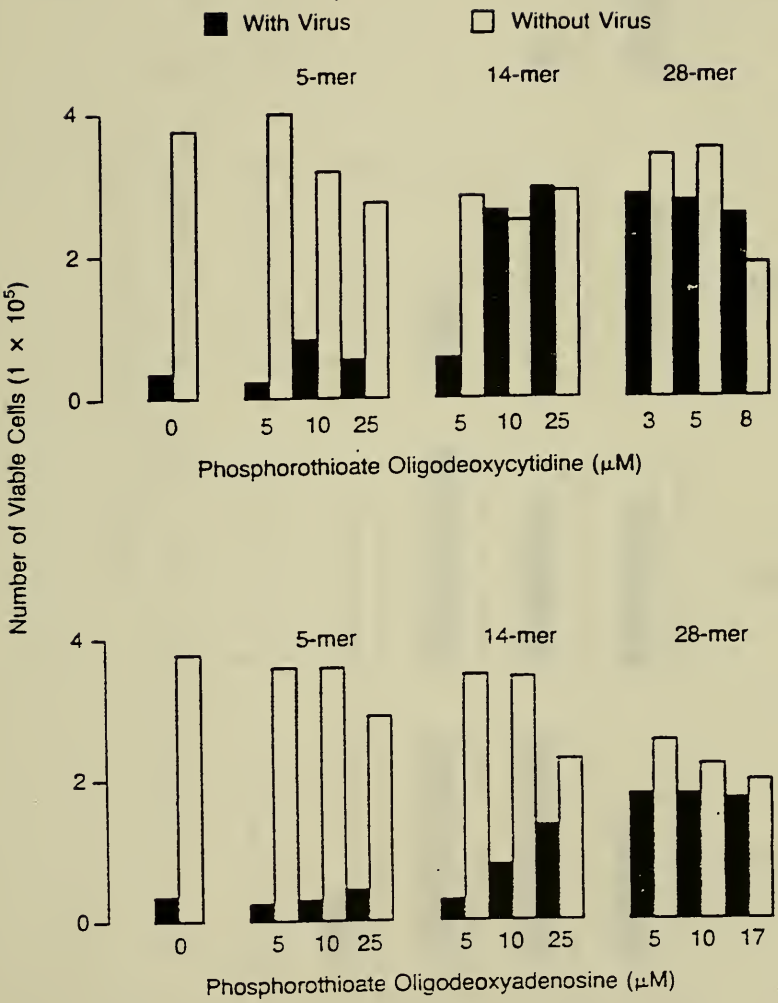
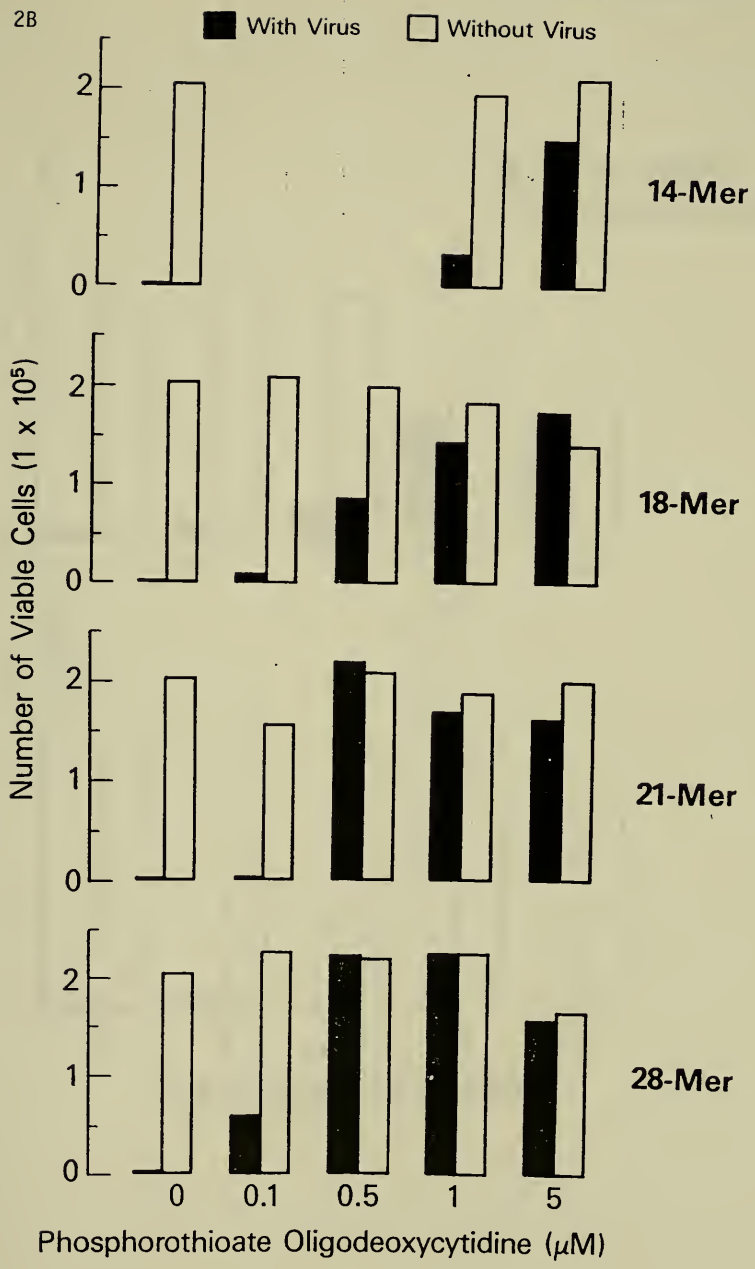

With Virus
Without Virus
5-mer
14-mer
28-mer
Number of Viable Cells (1 × 10⁵)
4
2
0
0
5 10 25
5 10 25
3 5 8
Phosphorothioate Oligodeoxycytidine (μM)
5-mer
14-mer
28-mer
4
2
0
0
5 10 25
5 10 25
5 10 17
Phosphorothioate Oligodeoxyadenosine (μM)

Fig. 2B


With Virus
Without Virus
14-Mer
18-Mer
21-Mer
28-Mer
Number of Viable Cells (1 x 10⁵)
0
1
2
0
0.1
0.5
1
5
Phosphorothioate Oligodeoxycytidine (μM)

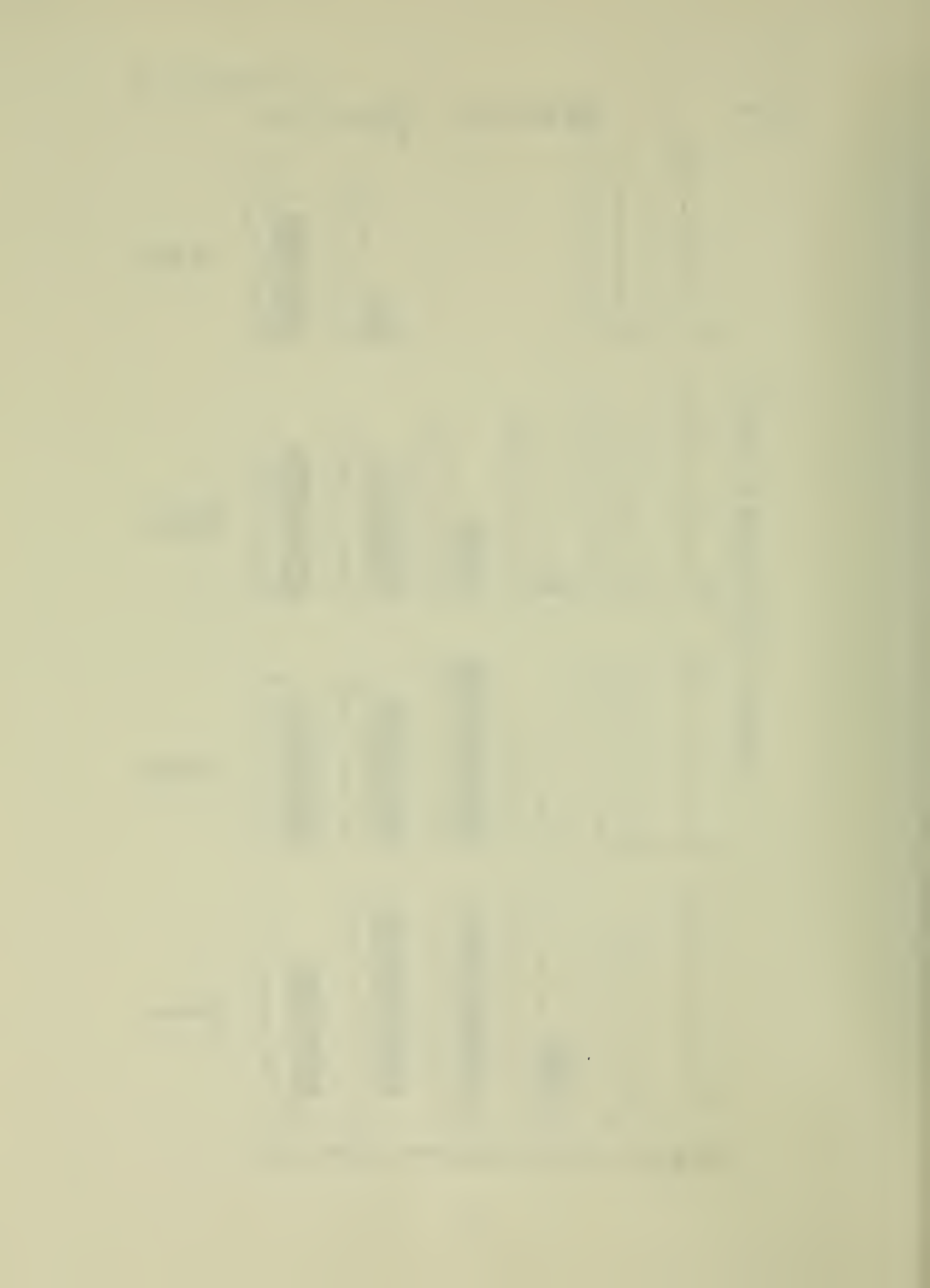


Fig. 3

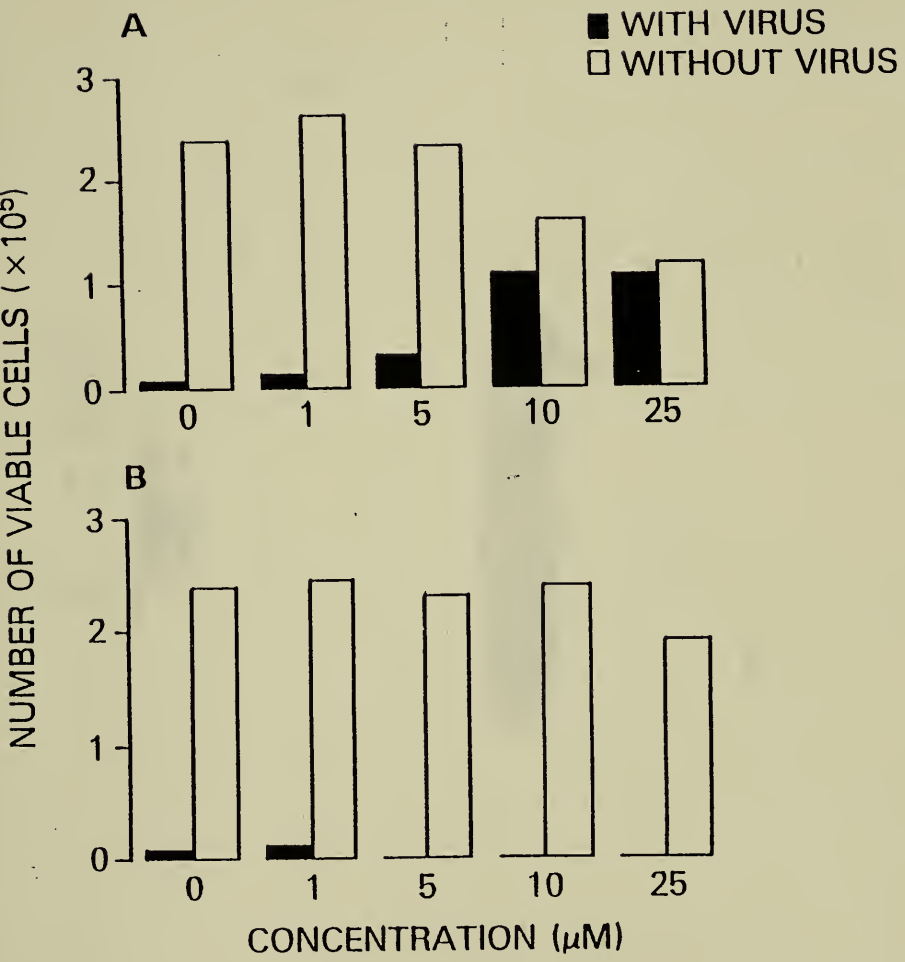

A
WITH VIRUS
WITHOUT VIRUS
3
2
1
0
0
1
5
10
25
B
3
2
1
0
0
1
5
10
25
NUMBER OF VIABLE CELLS (×10⁵)
CONCENTRATION (μM)

Fig. 4

Phosphorothioate (S-dC$_{28}$) in Chronically HIV-Infected T-Cells.

As illustrated in Table 2, S-dC$_{28}$ failed to reduce gag protein positivity of target cells assessed by indirect immunofluorescent assay in chronically HIV-infected H9 cells at concentrations as high as 25uM for the duration of the experiment (120hr).

Synergistic Enhancement of Antiviral Activity of 2',3'-Dideoxyadenosine by 14-mer Oligodeoxycytidine Phosphorothioate.

It is worth stressing that the various dideoxynucleosides (including azidothymidine (AZT), dideoxycytidine, and dideoxyadenosine) require anabolic phosphorylation within target cells to become active anti-retroviral agents. The mechanisms of action appear to be competitive inhibition of reverse transcriptase and/or termination of nascent DNA chain formation (see discussion in accompanying report). The oligomers under discussion are likely to work by different mechanisms and would not be expected to require anabolic phosphorylation. It was, therefore, of interest to see if we could observe synergistic anti-viral effects when we combined an anti-viral oligomer with a dideoxynucleoside. The combination of 2',3'dideoxyadenosine (ddAdo) and 14-mer oligodeoxycytidine phosphorothioate gave a marked synergistic enhancement of antiviral activity (Fig.5). For example, 2uM of ddAdo showed complete protection of target cells against the viral cytopathic effect with 5uM S-dC$_{14}$, while each of the two agents alone showed very marginal protective effects in this experiment.

Discussion

Since only phosphorothioate analogs showed anti-HIV activity (Table 1) in our assay we presume that it is mainly the relative resistance of the phosphorothioate analogs to nucleases that preserves them relative to n-ODNs, and allows them to reach and remain at their target site. This was supported in relation to the media used in our in vitro test system by following the ^{31}P NMR spectra of the n-ODN-1 and S-OND-1 compounds as a function of time. Breakdown of the normal oligodeoxynucleotide was seen from the build-up of the terminal phosphate peak, indicating a half-life of ca. 17 hrs. under these conditions, while the S-analogs exhibited no significant degradation even after a week (within the accuracy of the method <5%). Similarly, samples of solutions of S-ODNs taken from our in vitro cytopathic assay and incubated in human serum at 37^{0}C showed no degradation after 7 days (data not shown). The inactivity of a methylphosphonate analog (M-ODN-1) in our cytopathic inhibition assay could have been due to its poor ability to hybridize strongly to the target sequence.

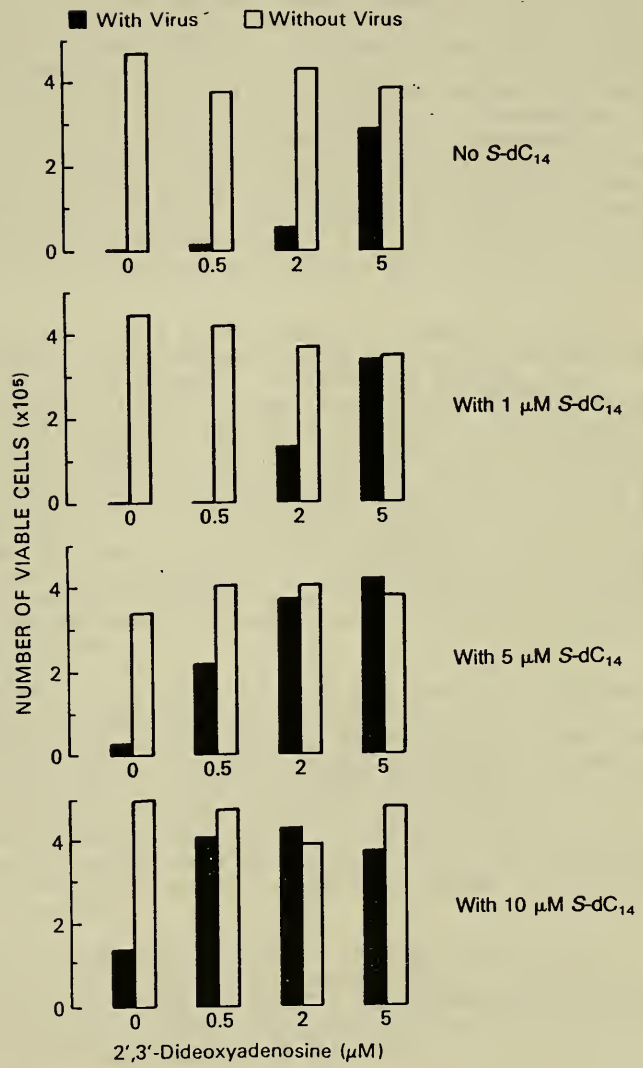
The potency of anti-HIV activity of S-dC$_{28}$, one of most potent analogs tested, is almost comparable to that of 2',3'-dideoxycytidine(ddCyd) on the basis of molarity (both agents showed complete antiviral activity at 0.5uM in our assay system); and also in terms of therapeutic index(ratio of cytotoxic concentration to effective concentration), S-dC$_{28}$ generally shows a comparable in vitro index to those of ddCyd and ddAdo(i.e.,10 to 20).

Generally it has been assumed that anti-sense sequences inhibit the

Table 2

S-dC$_{28}$	Percentage of gag positive cells (Hours in Culture with Compound)			
	8	24	72	120
0uM	79	90	70	78
5uM	82	91	85	79
10uM	74	80	71	82
25uM	69	86	75	74

Fig. 5


With Virus
Without Virus
No S-dC14
With 1 μM S-dC14
With 5 μM S-dC14
With 10 μM S-dC14
NUMBER OF VIABLE CELLS (x10⁵)
0
2
4
0
0.5
2
5
2',3'-Dideoxyadenosine (μM)

expression of various genes by translation arrest, i.e. that they bind to mRNA and block its translation. In order to test this possibilty we analysed gag protein synthesis in chronically HTLV-III$_B$-infected and -producing H9 (H9/III$_B$) cells by indirect immunofluorescent assay under a microscope. S-dC$_{28}$ did not inhibit gag protein positivity in H9/III$_B$ cells at concentrations as high as 25uM (Table 2). Although gag positivity of cells is only a partially quantitative parameter for protein production, this result suggests that the potent anti-HIV activity of S-dC$_{28}$ at concentration as low as 0.5 uM might not be from a translation arrest per se. Alternatively, the level of any translation arrest could have been below our threshold of detection by indirect immunofluorescent assay under a microscope. By contrast, a Southern blot analysis employed to explore de novo synthesis of HIV DNA in target cells showed complete inhibition by S-dC$_{28}$ at concentrations down to 1uM (Fig.4). Therefore, one mechanism for the antiviral effect could depend on blocking viral replication perhaps prior to, or at the stage of, pro-viral DNA synthesis.

We tested the possibilty that the S-ODN analogs may interfere with HIV binding to target cells. The T4 molecule on the cell surface is known to be the main receptor for HIV in T4$^+$ cells. No inhibition by S-dC$_{28}$ was observed in experiments using radiolabelled virus for specific binding of the labelled virus to the T4 molecule in T4$^+$ cells (H9 cells), thus suggesting that inhibition of viral binding to the cell surface is not responsible for the activity (D. Looney, personal communication). In addition, no detectable changes in the T4, HLA-DR, T8, T3 or Tac antigen on the cell surface of ATH8 cells were shown by fluorescent-activated cytofluorometry after 16 hr incubation with 1uM S-dC$_{28}$ (data not shown). Overall these findings, including a base composition effect and a length effect (Table 1 and Fig.2A and Fig.2B) suggest that the antiviral activity is mediated by inhibition of HIV pro-viral DNA synthesis, perhaps brought about, at least in part, by binding of the S-ODNs to a viral nucleotide sequence.

Another mechanism to be considered is induction of interferon production such as that proposed for phophorothioate analogs of poly-r(I-C). No induction of gamma-interferon was observed in the supernatant of the culture with S-dC$_{14}$, and 1,000 units of recombinant alpha- or gamma-interferon added directly to the cultures did not inhibit the cytopathic effect in our assay system. Also since there are no data to support the concept that phosphorothioate internucleotide linkages have a thiol character, and can thus form disulfides, the mechanism of action would likely be different from that proposed for antiviral polynucleotides having thiolated bases such as 5-mercapto-cytosine or -uracil.

Phosphatase-resistant ^{35}S-phosphorothioate end-labelled S-dC$_{28}$ was employed to investigate the permeability of target cells. Significant increases of radioactivity in ATH8 and H9 cells were observed within several minutes(unpublished data) as reported previously for normal ODNs, thus supporting the uptake of these compounds by the cells.

S-ODNs also showed substantial inhibition of purified HIV reverse transcriptase activity in the in vitro experiment using a viral DNA (3'-orf) inserted in an M-13 vector as a template with universal primer under

previously described conditions. Under some conditions, phosphorothioate analogs can serve as competitive inhibitors of templateprimer in some cases with a Ki of 3nM and this class of compounds appears to have multiple mechanisms of action. The precise mechanism(s), however, including nonsequence specificity of the antiviral activity, direct inhibition of the viral DNA polymerase or additional translation arrest at high concentration for complementary sequences require further research at this time. In recent studies we have observed that high (but non-toxic) concentrations of phosphorothioate analogues in a complementary (anti-sense) configuration to art-gene mRna can inhibit viral expression in chronically infected cells. Irrespective of the precise mechanism the anti-HIV effect of these agents is potentially important from both a clinical and theoretical point of view.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 07202-04 BDMS

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biostatistics and Data Management Section

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

P.I.:	Seth M. Steinberg	Acting Head	BDMS, COP, DCT, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION
Biostatistics and Data Management Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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.)*

The Section is the statistical and data management component of the Clinical Oncology Program (COP). The Section provides statistical leadership and data management consultation for major activities of the Program, and is involved in the design, conduct, monitoring, and statistical analyses of intramural and national multicenter clinical trials of experimental treatments for cancer. Other major collaborative efforts include studies to identify important prognostic and treatment selection factors, evaluate diagnostic procedures, develop improved staging systems, and assist investigators in the design, execution, and analyses of major in vitro drug testing studies. The Section develops new statistical designs and biometric methods related to the development and evaluation of new cancer treatments. The Section maintains computerized data collection systems for intramural and national multicenter clinical protocols, and it works closely with interested branches to improve data recording and retrieval. The Section works with the Clinical Center Medical Information System team, allowing COP input into decisions which directly impact patient care and protocol management. The Section assists the Deputy Clinical Director to insure adequate monitoring of protocols through the MIS Toxicity screens and other mechanisms.

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

1. Collaborative Projects Within Clinical Oncology Program

Members of the Biostatistics and Data Management Section provide to the intramural clinical research program both biostatistical and data management expertise. Our efforts in these areas are described in sections A) and B) below.

A. The Biostatistics and Data Management Section (BDMS) is organized with a designated coordinating statistician for each Clinical Oncology Program (COP) branch. A member of the BDMS participates in the development of new protocols and the interim monitoring and data collection for ongoing studies. A member of the Section also serves on the Clinical Research Sub-Panel to review all intramural clinical trials. BDMS staff collaborate in clinical and laboratory studies to evaluate prognostic and treatment selection factors and elucidate tumor biology. The Section provides statistical support for the COP as well as advice on the best ways to use available NIH computer systems or microprocessor based professional workstations for clinical and laboratory research.

A detailed list of COP projects to which members of the Section have provided statistical input follows:
(1) Performed two interim analyses of ALL (leukemia) protocol 77-02, a cooperative study with 181 patients at five institutions.
(2) Performed two interim analyses of two ALL protocols for average and high risk patients; the high risk protocol is a single arm extension of the successful chemotherapy only (no cranial irradiation) arm on the multi-institutional 77-02 protocol, with modifications in Ara-C administration to prevent CNS relapse and to more aggressively treat systemic disease. The average risk protocol is a randomized extension of 77-02, comparing two chemotherapy only arms - one with high dose methotrexate and one without.
(3) Performed three interim analyses of four phase II trials of 6MP in 85 patients at over a dozen institutions participating in the Pediatric Oncology Group.
(4) Arranged for randomizations and eligibility checklists for protocols to be conducted through Surgery Branch and NCI-Navy Medical Oncology Branch.
(5) Provided advice to consultants responsible for psychological evaluation of patients on Pediatric Branch protocols.
(6) Served as member on Institutional Review Board.
(7) Performed major update of results on all soft tissue sarcoma protocols, comparing adjuvant chemotherapy to no chemotherapy in patients with extremity tumors, and with head, neck and trunk tumors, comparing limb-sparing surgery to amputation in patients receiving adjuvant chemotherapy, comparing a short course adjuvant chemotherapy regimen (350 mg/m^2 doxorubicin) with standard course (550 mg/m^2); and comparing radiation to no radiation in patients with high grade soft tissue sarcoma of extremities with local surgical resection or with low grade soft tissue sarcoma of head, neck and truck, or extremities.
(8) Analyzed data from experiments examining effects of PTH, Indomethacin, EGF, and conditioned media on bone resorption.
(9) Analyzed data evaluating the utility of radioimmunoassay for serum CA 19-9 levels in patients with carcinoma of the pancreas.
(10) Determined sample sizes and stratification scheme for a randomized trial of IL-2/LAK in patients with renal cell cancer.

(11) Performed analyses of renal toxicity data from IL-2/LAK protocols.
(12) Determined sample sizes needed for a protocol involving protochemotherapy of advanced stage limited non-small cell lung carcinoma.
(13) Analyzed data on prognostic factors in esophageal cancer.
(14) Analyzed data regarding relative abilities of CT and NMR (at three different settings) to contrast tumor with different types of surrounding tissue.
(15) Provided advice regarding analysis of data from a study of peripheral neuropathy following cisplatin administration.
(16) Provided review for statistical considerations of two manuscripts being considered for publication in Blood.
(17) Performed analysis of data for a study dealing with pain following amputation of a limb.
(18) Performed analysis of metastatic breast cancer protocol.
(19) Analyzed data relating concentration of drug to achieve ID50 with characteristics of patients and cell lines taken from patients.
(20) Determined statistical considerations for a randomized study to treat mycosis fungoides limited to skin and lymph nodes.
(21) Performed analysis of data regarding MTT assay technique.
(22) Evaluated the plausibility of switching to a pre-randomization scheme for a slowly accruing study of patients with early stage breast cancer.
(23) Analyzed data for a study of rehabilitation among patients being treated on the early stage breast cancer protocol.
(24) Prepared and delivered a lecture on statistical considerations in the design and analysis of cancer clinical trials.
(25) Performed analysis of data on a study evaluating effects of perioperative transfusions in patients with colorectal hepatic metastases.
(26) Performed analyses of data relating effects of renal cell cancer on renal toxicity in patients treated on IL-2/LAK protocols.
(27) Analyzed data to determine whether response to therapy for ovarian cancer can be predicted on the basis of characteristics of the patients and the tumor.
(28) Performed analysis of data from study relating to treatment of AIDS patients.
(29) Analyzed data from study comparing intravenous vs. intraarterial infusion of FUDR for treatment of hepatic metastases in colorectal cancer.
(30) Performed analysis of data for the locally advanced breast cancer study.
(31) Determined potential impact of additional accrual to the extensive small cell lung cancer protocol.
(32) Prepared statistical considerations section for a phase II study of 2'-Deoxycoformycin in patients with hairy cell leukemia or T gamma lymphoproliferative disease refractory to or intolerant of low dose alpha interferon.
(33) Performed analysis of data from a study of patients with malignant pheochromocytoma.
(34) Analyzed data pertaining to percent conjugation.
(35) Provided consultations to investigators analyzing data from studies of combined modality therapy in early stage aggressive lymphoma.
(36) Refereed a paper submitted for consideration by Archives of Internal Medicine.
(37) Performed analysis of data from a study evaluating cosmesis in the early breast cancer study.
(38) Analyzed data from retrospective study of stage I and II indolent lymphoma.
(39) Performed analysis of data identifying prognostic importance of tumor

pathology in patients with soft tissue sarcomas.
(40) Analyzed data for study of mycosis fungoides.
(41) Performed analysis of prognostic factors in patients with diffuse histiocytic lymphoma.
(42) Participated in workshop on design of software for clinical trials and laboratory studies.
(43) Participated in discussions with respect to design of a retrospective study to determine whether patients with cancer admitted to the Clinical Center ICU have outcomes that are consistent with those of non-cancer patients admitted to the ICU.
(44) Performed analyses of prognostic factors in patients treated for small cell lung cancer.
(45) Performed analyses of quality of life data for patients who received therapy for soft tissue sarcomas.
(46) Performed analyses to determine whether extrahepatic disease can be predicted in patients.
(47) Provided advice regarding analysis of data from a study of bone growth in rats.
(48) Prepared statistical considerations for a randomized trial to determine the validity of criteria for the addition of vancomycin to ceftazadime in the management of febrile granulocytopenic patients.
(49) Identified sample sizes needed for testing differences between proportions of responders among patients who do or do not have expression of a particular gene.
(50) Analyzed data for extensive small cell lung cancer protocol.
(51) Performed analysis of drug sensitivity in lung cancer patients.
(52) Developed scheme for randomization in protocol testing of oxacillin in catheters for IL-2 administration.
(53) Performed analyses of prognostic factors in survival among patients on the randomized protocol comparing intravenous vs. intraarterial FUDR for treatment of hepatic metastases from colorectal cancer.
(54) Prepared analysis of non-small cell lung cancer protocol data.
(55) Performed analyses of chemosensitivity of human colorectal carcinoma cell lines.
(56) Prepared statistical considerations for a protocol of treatment for advanced or refractory mycosis fungoides.
(57) Performed analyses of data from the randomized early breast cancer study.
(58) Provided consultation regarding determination of lytic units.
(59) Performed analyses to predict treatment outcome for patients with fever.
(60) Performed analysis of data from metastatic breast cancer study.
(61) Performed analysis of data from a study of patients with osteosarcoma.
(62) Performed analyses to identify factors influencing leucovorin and 5-FU sensitivity in colon cancer cell lines.
(63) Performed analyses of data pertaining to tumor shrinkage in animals following administration of various drugs.
(64) Performed analyses to identify factors which may be associated with response, and with response duration, in patients who have advanced renal cell cancer or melanoma, and who have been treated on the IL-2/LAK protocols.
(65) Provided consultation regarding methods of analysis to demonstrate associations between sensitivities to drugs in cell lines, in successive experiments, and regarding methods for testing for differences among groups of lung cancer

cell lines.
(66) Provided consultation regarding sample sizes for a retrospective study to estimate proportions of male breast cancer patients who have a familial tendency towards the disease.
(67) Researched the possible utility to the COP of gaining access to the Frederick Cancer Research Facility VAX computer.
(68) Provided consultation to determine whether infants differ from children with respect to proportions with gene rearrangements.
(69) Prepared statistical considerations for randomized head and neck treatment protocol.
(70) Participated in discussions regarding a study of effects of craniospinal irradiation on children who have experienced a CNS relapse while being treated for ALL, and performed preliminary analyses.
(71) Provided advice regarding uses of personal computer software for analysis of small data sets.
(72) Performed analyses of toxicity data from a study of childhood ALL.
(73) Provided consultation regarding statistical considerations for a study of Mesna in patients receiving cyclophosphamide.
(74) Performed analyses of data from a study of patients who received continuous infusion IdUrd for glioblastoma.
(75) Participated in discussions with COP and Clinical Center personnel conducting a survey regarding confidentiality of hospital records.
(76) Participated in discussions regarding data collection and forms design for a study of effects of radiation therapy on patients with soft tissue sarcomas of the extremities.
(77) Provided consultation regarding design of a study to evaluate the cardiac toxicity of patients being treated with radiation for locally advanced non-metastatic breast cancer.
(78) Provided advice regarding data from a breast cancer study of nausea and vomiting.
(79) Performed analysis of male breast cancer study.
(80) Performed analyses of data correlating pathologic findings, clinical information, and outcome in patients with lung cancer.
(81) Participated in Surgery Branch end-of-year protocol reviews.
(82) Determined significance of difference in complication and infection rates for patients on the Hickman catheter vs. Port-a-cath study.

B. Data Management Activities

The Section has continued the development and maintenance of several systems which facilitate the monitoring of protocols. (1) A comprehensive data management system was developed for monitoring all IL-2/LAK protocols. (2) The CAPRI system, the primary COP database, has been continuously updated. (3) An assessment of the overall COP data management needs, branch by branch, has been completed. (4) A variety of graphs, plots and other statistical reports have been provided to COP branches. (5) In support of the COP use of personal computers, provided assistance and consultation in the selection and installation of hardware, evaluation of software packages, and designing and implementing specific PC programs.

A detailed list of data management projects undertaken by members of the BDMS

for the COP follows:
(1) Collection, maintenance, and reporting of basic survival and protocol entry data on COP active patients.
(2) Data management, programming, retrievals and analyses as required by Surgery Branch staff.
(3) Data collection and coordination for several current protocols of the NCI-Navy Medical Oncology Branch.
(4) Maintenance of a computer file of the latest actual outpatient clinic visit for all active patients.
(5) Support to insure that all patients receiving chemotherapy, especially investigational drugs, have a valid Clinical Center protocol number for pharmacy records.
(6) Phase II studies annual toxicity report.
(7) Maintenance of various computer packages used by the Section.
(8) Collaboration with Clinical Center computer staff on abstracting MIS data for protocol uses, and on computerized hospital data collection.
(9) Automatic printing of all data from the MIS Toxicity and Protocol Monitoring System as Progress Notes and distribution of these documents to appropriate investigators.
(10) Assist data nurses and principal investigators in using personal computers for protocol data management.
(11) Provide branches with access to computer programs to do standard statistical analyses.
(12) Evaluation of software packages for the IBM-PC.
(13) Maintain major statistical and plotting programs for PC to allow BDMS statisticians to perform Kaplan-Meier curves, logrank tests (including stratified version), logistic regression, and Cox regression.
(14) Computer needs analysis and equipment and software purchase recommendations for acquiring personal computers; training staff in the use of PC's.
(15) Provided data management support for all IL-2/LAK protocols through initiation and/or completion and maintenance of the nine computer forms using prospective and retrospective review of office charts and medical records.
(16) Determined completion status of each of the nine computer forms for all IL-2/LAK protocols and manually provided lists of those patients to the staff responsible for completing the forms.
(17) Assisted the senior staff in measuring and recording evaluable tumors for all IL-2/LAK patients.
(18) Submitted for data entry all IL-2/LAK forms, proofread and corrected all data entered, and provided updates of the IL-2/LAK Master List.
(19) Designed, conducted and reported on the assessment of the short- and long-term data management needs of each COP branch.
(20) Provided extensive support to the Radiation Oncology Branch and the Early Stage Breast Cancer Protocol, including the preparation of programs, plots, graphs and reports.
(21) Continued support of COP randomization activities with the addition of four new protocols and the revision of several existing studies.
(22) Revised and modified the Travel Order and Voucher System, and developed a Geographic Distribution Report for the administrative office of the COP.
(23) Provided data management support to the Surgery Branch for both an FDA-sponsored Theradex audit and a subsequent FDA field inspector audit.
(24) Developed patient tracking systems for the Pediatric and NCI-Navy Medical

Oncology Branches.
(25) Continued to support the implementation of the Memorial-Sloan Kettering System on the Data General at the NCI-Navy Medical Oncology Branch, including training of personnel, system maintenance and documentation.
(26) Acted as a coordinating center for four multi-institutional clinical trials in ovarian cancer and pediatric leukemia, involving data processing, analysis and report production.

2. Projects Outside COP

A. The BDMS also participates in biometric activities outside of the COP. A detailed list of projects outside of COP in which the Section's statistician has provided statistical input include the following:

(1) Analysis, advice, and consultation on extraspinal tendon and ligament calcification associated with chronic etretinate therapy (for Dermatology Branch of the Division of Cancer Biology and Diagnosis, NCI).
(2) Consultation regarding analysis of data pertaining to swelling of sperm tails in two different assays (for NICHD).
(3) Consultation and analysis of data for a study of cardiac output after septic shock and administration of endotoxin (for Clinical Center Critical Care Medicine Department (CC-CCM)).
(4) Consultation regarding a study of islet cell carcinoma (for NIDDK).
(5) Analysis of data in order to characterize morphology of normal human sperm, and to establish standards by which to evaluate whether sperm of infertile humans differs significantly from those of fertile humans (for NICHD).
(6) Provide advice regarding use of statistical tests in evaluating serial data collected from patients in the intensive care unit (for CC-CCM).
(7) Determination of sample sizes for an experiment to demonstrate that addition of heparin to a catheter results in equivalent patency as that from saline alone (for CC-CCM).
(8) Reviewed and revised a questionnaire regarding pain palliation (for Clinical Center Pharmacy Department).
(9) Provided advice regarding questionnaire design for a study of fatigue in arthritis (for Clinical Center Rehabilitation Medicine Department).
(10) Provided advice regarding statistical considerations for a study of pain relievers for cancer patients (for NIDR).

B. In addition to data management support for intramural trials, the BDMS provides data management services outside the COP. Project staff have provided Operations and/or Statistical Center support to a number of multi-institutional extramural trials. This support includes performing randomizations, design of data collection instruments, software design and development, production of regular status reports, and production of ad hoc reports and tabulations as directed by the study statistician. The extramural trials supported include:
(1) 7601/7602, Treatment of Early Stage Ovarian Cancer
(2) CCSG-191P, CCSG Protocol for Acute Lymphoblastic Leukemia
(3) CCSG-134P, CCSG Protocol for Poor Prognosis Acute Lymphoblastic Leukemia
(4) CCSG-144P, CCSG Protocol for Average Prognosis Acute Lymphoblastic Leukemia

3. Biometric Research

Current biostatistical research being conducted includes:
(1) Use of distribution-free methods for estimation of interquantile distances.
(2) Non-parametric and parametric approaches to quantile estimation.
(3) A two stage method for selecting interactions between variables to be evaluated for prognostic importance.
(4) Development of data management systems which may serve multiple purposes.

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2. Chang, A.E., Matory, Y.L., Dwyer, A.J., Hill, S.C., Girton, M.E., Steinberg, S.M., Knop, R.H., Frank, J.A., Hyams, D., Doppman, J.L. and Rosenberg, S.A.: Magnetic resonance imagery versus computed tomography in the evaluation of soft tissue tumors of the extremities. Annals of Surgery 205: 340-348, 1987.

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5. Douglass, H.O. Jr., Kalser, M.H., Stablein, D.M. and Steinberg, S.M.: Further evidence of effective adjuvant combined radiation and chemotherapy following curative resection of pancreatic cancer. Cancer (in press).

6. Douglass, H.O. Jr., Stablein, D., Kalser, M., Weaver, D., Marsh, J., Woolley, P., Bruckner, H., Levin, B., Knowlton, A. and Steinberg, S.: Confirmation by the Gastrointestinal Tumor Study Group that survival following potentially curative resection of pancreatic cancer is improved by multidisciplinary post-operative therapy. In Proceedings of Fifth Adjuvant Therapy of Cancer Conference, Grune and Stratton (in press).

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function in patients receiving immunotherapy for advanced cancer. Annals of Internal Medicine (in press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-CM-06513-11-CP

PERIOD COVERED: October 1, 1986, to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Molecular Pharmacology of Antitumor Agents

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

Bruce A. Chabner, M.D., Director, DCT	OD, DCT, NCI
Gregory A. Curt, M.D., Deputy Director, DCT	OD, DCT, NCI
Keisuke Aiba, M.D., Guest Researcher	CPB, DCT, NCI
Carmen J. Allegra, M.D., Medical Staff Fellow	CPB, DCT, NCI
Jacob Baram, M.D., Visiting Fellow	CPB, DCT, NCI
Robert L. Fine, M.D., Medical Staff Fellow	CPB, DCT, NCI
Donna Boarman, Biologist	CPB, DCT, NCI
James C. Drake, Biologist	CPB, DCT, NCI

COOPERATING UNITS *(if any)*

NCI-Navy Medical Oncology Branch, COP, DCT, NCI.
Critical Care Medicine Department, Clinical Center, NIH.

LAB/BRANCH: Clinical Pharmacology Branch

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	4.0	2.0

CHECK APPROPRIATE BOX(ES)

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

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

During Fiscal Year 1987 our work continued on the mechanism of action of antifolates. Our major projects were the following:

1. Continued work on the mechanism of de novo purine and thymidylate synthesis inhibition by antifolates, including methotrexate, has strengthened a novel postulate that inhibition of metabolic pathways results from direct enzyme inhibition rather than via an indirect mechanism of folate depletion. These studies illustrate that de novo purine and thymidylate synthesis are inhibited by clinically relevant concentrations of methotrexate without significant folate depletion in two human cell lines (breast MCF-7 and promyelocytic HL-60) and in normal human myeloid progenitor cells harvested and purified from normal human volunteers. These studies further suggest that the mechanism of metabolic inhibition is through the direct inhibitory effects of accumulated dihydrofolate polyglutamates on the folate-requiring enzymes thymidylate synthase and AICAR transformylase.

2. During the course of the above studies, a novel physiologic folate was discovered, 10-formyl-dihydrofolate. Continued investigations have illustrated the formation of this compound in breast cells and in normal human myeloid progenitor cells. Identification and characterization of the enzyme responsible for the formylation of dihydrofolate has been accomplished and the effects of 10-formyl-dihydrofolate on critical folate-requiring enzymes have been investigated. The new folate is a potent inhibitor of thymidylate synthase and GAR transformylase but, surprisingly, is an excellent substrate for AICAR transformylase, further

supporting the concept that folate depletion is only a minor factor in the mechanism of action of methotrexate.

3. Drug resistance to antimetabolite agents is a major limiting factor in the therapy of malignant diseases. We have investigated the interaction of leucovorin with 5-fluorouracil in multiple colon cell lines developed by Dr. Park at the NCI-Navy Medical Oncology Branch. We have found that leucovorin can markedly enhance the potency of 5-fluorouracil and 5-fluorodeoxyuridine in selected lines. Currently, we are investigating the molecular mechanism of this interaction and we are investigating the additional mechanisms of resistance in the setting of combination treatment with fluoropyrimidines and leucovorin. Parallel studies are ongoing using tumor samples from patients with breast cancer who are being treated with 5-fluorouracil and leucovorin.

4. Pneumocystis carinii (PC) pneumonia is the most commonly recognized cause of mortality in AIDS patients. Traditional therapy includes the use of the antifolate trimethoprim. Clinically resistant infection and adverse reactions to standard therapies occur in up to 65% of AIDS patients being treated for PC pneumonia. Our laboratory has developed a novel therapy for this infection using trimetrexate, a lipid-soluble antineoplastic antifolate that was found to be 1500-fold more potent than trimethoprim in inhibiting the catalytic activity of PC dihydrofolate reductase. We further found that leucovorin can easily penetrate mammalian host cells and effect rescue of these cells from the lethal effects of trimetrexate. PC organisms are unable to transport leucovorin and are thus killed by the trimetrexate. These findings were applied to the treatment of PC pneumonia in 49 AIDS patients. The overall response rate, including patients failing conventional therapy, was 70%. We feel that the use of trimetrexate with leucovorin represents an important, novel approach to the treatment of this highly lethal and prevalent infection.

5. Recent work has demonstrated that multidrug-resistant human breast and small-cell lung cancer lines have an increased phosphorylation of a 20-kD membrane-bound protein. Evidence suggests that protein kinase C is responsible for the phosphorylation, and we have found its activity to be sevenfold higher in resistant breast cancer cells. When protein kinase C is activated, it leads to increased multidrug resistance and phosphorylation of the 20-kD protein as well as decreased intracellular drug accumulation. This induced multidrug-resistant phenotype can be inhibited by calcium channel blockers and calmodulin antagonists. We are investigating protein kinase C inhibitors, such as staurosporine and bryostatin, for activity against resistant tumor cell lines. Concomitant with our studies on protein kinase C, we are looking at the phosphatidylinositol (PI) cycle and have found increased activity of this cycle in resistant cells. We have also found increased arachodonic acid metabolism, and we are attempting to exploit these differences with agents known to inhibit the PI cycle (plant PI) and prostaglandin and leukotriene pathways (SRS-A analogs).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06516 06 CP

PERIOD COVERED October 1, 1986 to September 30, 1987

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

Drug Resistance in Human Tumor Cells

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

Kenneth H. Cowan, M.D., Ph.D.	Senior Investigator	CPB, COP, DCT, NCI
Merrill E. Goldsmith, Ph.D.	Microbiologist	CPB, COP, DCT, NCI
Craig Fairchild, Ph.D.	PRAT Fellow	CPB, COP, DCT, NCI
S. Percy Ivy, M.D.	Biotechnician	CPB, COP, DCT, NCI
Philip Vickers, M.D.	Visiting Fellow	CPB, COP, DCT, NCI
Alan Townsend, M.D.	Biotechnician	CPB, COP, DCT, NCI
Jeffrey Moscow, M.D.	Clinical Associate	CPB, COP, DCT, NCI
Mary Jane Madden, M.D.	Chemist	CPB, COP, DCT, NCI

COOPERATING UNITS *(if any)*

Medicine Branch, COP, DCT, NCI
BRMP, FCRC, NCI

LAB/BRANCH Clinical Pharmacology Branch

SECTION Office of the Chief

INSTITUTE AND LOCATION NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

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

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

This labortatory has been investigating genetic and biochemical changes associated with drug resistance in human tumors. We have characterized an adriamycin resistant human breast cancer cell line which has developed the phenotype of multidrug resistance. Resistance is associated with decreased drug accumulation (2-3 fold) increased activities of glutathione peroxidase (12 fold), glutathione transferase (45 fold), decreased expression of aryl hydrocarbon hydroxylase (cytochrome P1-450). We have isolated cDNA clones from this resistant cell line which encode the gP 170 membrane glycoprotein, a gene which is often, if not always, associated with the development of multidrug resistance. We have also cloned the cDNA for the anionic glutathione transferase which is transcriptionally activated in the Adr^R MCF-7 cells. We have begun a study investigating the expression of the P-glycoprotein gene as well as the expression of several drug metabolizing enzymes including the anionic glutathione transferase GST-π in the development of clinical drug resistance.

A. Multidrug Resistance in Human Breast Cancer Cells

We have been studying the molecular genetic and biochemical changes in multidrug resistant breast cancer cells selected in our laboratory. Resistance in these cells is associated with 2-3 fold decrease in drug accumulation, increased expression of glutathione peroxidase and glutathione transferase activity, and decreased expression of aryl hydrocarbon hydroxylase (cytochrome P_1450). These changes are remarkably similar to those which are induced by carcinogens in rat hyperplastic liver nodules and which are associated with resistance to many structurally unrelated hepatotoxins in that system. In addition, an immunologically related aniomic glutathione transferase is induced in both MDR breast cancer cells and in xenobiotic resistant rat hyperplasic liver nodules. These studies suggest that the mechanisms associated with de novo and acquired drug resistance may be the same. Three manuscripts in this work have been published, two additional manuscripts have been submitted.

B. Glutathione S-Transferase

We have recently shown that multidrug resistance in MCF-7 breast cancer cells selected for primary resistance to adriamycin is associated with an increase in glutrothrone S-transferace activity. This icrease is due to the induction of the anionic isozyme of GST (GST π). We have purified this isozyme and generated polyclonal antibodies against it. Following affinity purification, this polyclonal antibody reacts with a single protein on western blot analysis. This antibody is being used to screen human tumor specimins for GST π expression using immunohistochemical staining and western blot analysis. Monoclonal antibodies against GST π purified from Adr^R MCF-7 cells have been generated in collaboration with Raphe Kantor at FCRF. These antibodies will be used to screen for different epitopes of anionic GST in order to compare the isozymes produced in different tissues.

Jeff Moscow has isolated GST π cDNA clones from a library constructed by Merrill Goldsmith from Adr^R MCF -7 RNA. This gene code for a 750 bp mRNA which is overexpressed in Adr^R cells. Mary Jane Madden has sequenced this gene and shown that its sequence is remarkably homologous to the rat anionic human GST π gene but differs markedly from the human basic GST genes.

The human GST π cDNA has been used to sreen RNA extracted from normal and malignant human tissues. GST π is expressed in 24/26 human colon cancers but in

only 2/10 normal colon tissue samples. This gene may thus represent a marker of de novo resistance to chemotherapy, in this carcinogen-induced tumor. Other studies have shown that GST π expression is increased in 2 patients with recurrent pre B cell ALL relative to the expression in 3 patients with pre B cell ALL at initial presentation. Thus, GST π expression may be a useful marker in acquired resistance to chemotherapy.

GST π expression also appears to correllate with the absence of estrogen receptors in primary breast cancer. GST π RNA is present in 6/6 ER negative ($\leq$10 fmoles lung) breast cancers but is present in low or undeductable levels in 5/6 ER positive breast cancers. Thus, this gene may be a marker of hormone resistance in breast cancer. Whether this reflects any difference in chemosenitivity is unclear. This work has been submitted for publication.

C. P- Glycoprotein - *mdr* gene

Craig Fairchild, and Percy Ivy, and Merrill Goldsmith have isolated P-glycoprotein cDNA sequences from Adr^R MCF-7 cells. This gene is overexpressed in most multidrug resistant cell lines. Its expression in human tumors is under investigation.

As alluded to previously, studies from our lab have shown that many biochemical changes in MDR MCR-7 cells are similar to those that are associated with xenobiotic resistance in carcinogen-induced rat hyperplastic liver nodules. Further studies have now shown that xenobiotic resistance in rat HNS and hepatomus is associated with overexpression of P-glycoprotein gene. Moreover, acute treatment with acetylaminofluorene and partial hepatectomy results in over an 80 fold induction of P-glycoprotein expression. This acute induction of P-glycoprotein represents an useful model to examine the reulation of P-glycoprotein gene expression. This work has been accepted for publication in the Proceedings of the National Academy of Sciences.

D. Alteration in Hormonal Sensitivity in MDR Breast Cancer Cells

MDR breast cancer cells have become cross resistant to antiestrogens. Phil Vickers in our lab in collaboration with Robert Dickson in the Medicine Branch has examined the hormonal sensitivity of Adr^R MCF-7 cells. In contrast to the parental MCF-7 cells, estrogen (E_2) does not increase, nor does the antiestrogen tamoxifen decrease the growth of Adr^R MCF-7 cells. Moreover, while E_2 induces secretion of specific polypeptides from parental MCF-7 cells and induces progesteron receptors in that cell

line, E_2 produce neither effect in Adr^R MCF-7 cells. This lack of hormone sensitivity of MCF-7 cells selected for adriamyecin resistance is associated with a loss of estrogen receptors as measured by hormone binding and antibody precipitation analysis. In contrast, membrane EGF receptors are markedly increased in the Adr^R MCF-7 cells. This increase in EGF receptors is associated with an increase in the responsivenes of the Adr^R MCF-7 cells to EGF (Dr. M. Jett and R. Fine, personal communication). What regulates this change in cytosolic and membrane receptors in cells selected for drug resistance is currently under investigation.

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Moscow, J., Myers, C.E., and Cowan, K.H., Acquired and Inherent Resistance toAntineoplastic Agents May Involve Identical Mechanisms. Cancer Treatment Reports, 1987 (manuscript in preparation).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-CM-06518-06-CP

PERIOD COVERED
Oct. 1, 1986 to Sept. 30, 1987

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

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

Jerry M. Collins, Ph.D.	Pharmacologist	CPB, COP, DCT, NCI
Raymond Klecker, B.S.	Chemist	CPB, COP, DCT, NCI
Raymond F. Greene, M.S.	Pharmacist	CPB, COP, DCT, NCI
Karl Belanger, M.D.	Visiting Fellow	CPB, COP, DCT, NCI
Paul Speth, M.D.	Guest Researcher	CPB, COP, DCT, NCI

COOPERATING UNITS *(if any)*

NCI/DCT/COP: ROB, SB; NCI/DCT/DTP; NCI/DCT/CTEP

LAB/BRANCH
Clinical Pharmacology Branch

SECTION
Pharmacokinetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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.)*

The primary focus of this project is always on the application of pharmacokinetic principles to questions of relevance to the treatment of cancer. The emphasis upon specific disease targets changes somewhat, as do the classes of drugs under investigation. The past year has continued trends towards emphasis of intracellular measurements in general, and pyrimidines in particular.

Over the last year, specific areas of interest included:

1. A clinical trial has been conducted with the Radiation Oncology Branch for the combined delivery of iododeoxyuridine and fluorodeoxyuridine. In addition to plasma pharmacokinetics, DNA from peripheral granulocytes has been studied for indications of biochemical modulation. This study will be completed when ongoing measurements of tumor DNA are completed. Most of our current efforts focus upon the hepatic arterial administration of iododeoxyridine as a cytotoxic agent as well as a radiosensitizer.

2. Phase I trials are an important milestone in the clinical pharmacology of anticancer drugs. The Blood Level Working Group is focusing specifically on the interface between preclinical and clinical studies.

3. Clinical pharmacokinetic studies have been completed for azidothymidine and are nearly finished for dideoxycytidine. It is anticipated that this is the final year for work on anti-AIDS drugs under this project.

(1) Halogenated Pyrimidines

In laboratory studies, we have developed the methodology to quantitate the replacement of thymidine in DNA with iododeoxyuridine (IdUrd). This methodology was first applied to peripheral granulocytes of patients receiving intravenous infusions of IdUrd. Replacement in DNA was correlated with myelosuppression. Next, the same methods were used to determine the modulation of IdUrd by simultaneous infusion of fluorodeoxyuridine. This clinical trial is nearly completed. We have started a trial of hepatic arterial infusion of IdUrd, and are applying the same methods to DNA obtained from tumor biopsies. We hope to correlate antitumor response with these DNA measurements. Initial clinical results have been very encouraging. Evidence of antitumor effect has been documented in each of the first 3 patients.

(2) Blood Level Working Group

Work on this project is accomplished mainly through collaborations with the extramural DCT staff at CTEP and DTP. Over the last 2 years, there has been significant progress in the integration of continuous infusion testing between preclinical and clinical studies. This coordination has resulted in a savings of 18 months in the clinical trial of deoxyspergualin and 12 months for merbarone, with a major reduction in the number of patients treated at biologically inactive doses. Work continues on the establishment of a relationship between species differences in toxicology and in metabolism. This aspect is being coordinated with the Pharmacokinetics and Metabolism Group of the European Organization for Research and Treatment of Cancer (EORTC). Based upon species differences in metabolism, the Phase I trials of several agents are currently using an accelerated dosing strategy.

(3) Evaluations of New Agents

A well-rounded approach to clinical pharmacology includes the exploration of new drugs. We have recently completed a Phase I clinical trial of flavone acetic acid. A variety of Phase I trials for new agents are currently being considered, including regional delivery approaches and drug combinations. The extent of our participation will depend upon the resources which are available.

(4) AIDS

In support of the DCT initiative for the treatment of AIDS, studies have been completed over the last 3 years on the clinical pharmacology of suramin, azidothymidine, and dideoxycytidine. Our group has developed assay methodology for all 3 drugs, characterized the clinical pharmacokinetics during initial human trials at NIH, and provided technical assistance for extramural trials utilizing these drugs. We now plan to return the focus of our work to full-time investigation of anticancer drugs.

PUBLICATIONS:

J.M. Collins, D.S. Zaharko, R.L. Dedrick, B.A. Chabner. Potential roles for preclinical pharmacology in Phase I trials. Cancer Treat. Rep. 70:73-80, 1986.

J.M. Collins, C.K.Grieshaber. Role of toxicology in anticancer drug development. Cancer Topics 6:38-39, 1987.

J. M. Collins, B. Leyland-Jones, C. K. Greishaber. Role of preclinical pharmacology in Phase I clinical trials: considerations of schedule-dependence. Concepts in Cancer Chemotherapy, F. M. Muggia (Ed.). Martinus Nijhoff. Boston. 1987. pp. 129-140.

R.W. Klecker, J.F. Jenkins, T.J. Kinsella, R.L. Fine, J.L. Strong, J.M. Collins. Iododeoxyuridine and iodouracil: Clinical pharmacology and modulation of endogenous pyrimidines. Clin. Pharmacol. Ther. 38:45-51, 1985.

K.Belanger, R.W.Klecker, J.Rowland, T.J.Kinsella, J.M.Collins. Incorporation of iododeoxyuridine into DNA of granulocytes in patients. Cancer Res. 46:6509-6512, 1986.

T.J.Kinsella, A.Russo, J.B.Mitchell, J.M.Collins, J.Rowland, D.Wright, E.Glatstein. Phase I study of intravenous iododeoxyuridine as a clinical radiosensitizer. Int. J. Radiat. Oncol. Biol. Phys. 11:1941-1946, 1985.

R. W. Klecker, J. M. Collins, R. Yarchoan, R. Thomas, J. F. Jenkins, S. Broder, C. E. Myers. Plasma and cerebrospinal fluid pharmacokinetics of 3'azido-3'-deoxythymidine: a novel pyrimidine analog with potential application for the treatment of patients with AIDS and related diseases. Clin. Pharmacol. Ther. 41:407-412, 1987.

J. M. Collins, R. W. Klecker, Jr., R. Yarchoan, H. C. Lane, A. S. Fauci, R. R. Redfield, S. Broder, C. E. Myers. Clinical pharmacokinetics of suramin in patients with HTLV-III/LAV infection. J. Clin. Pharmacol. 26:22-26, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06519 04 CP

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Non-Invasive Studies of Metabolism Using Nuclear Magnetic Resonance Methods

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

Jack S. Cohen, Ph.D.	Research Chemist	CPB, COP, DCT, NCI
Peter Daly, M.D.	Med. Staff Fellow	POB, COP, DCT, NCI
Robbe Lyon, Ph.D.	Sr. Staff Fellow	CPB, COP, DCT, NCI
Patrick Faustino, M.Sc.	Chemist	CPB, COP, DCT, NCI
Fredrique Megnin, M.Sc.	Guest Researcher	CPB, COP, DCT, NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Clinical Pharmacology Branch

SECTION: Biophysical Pharmacology Section

INSTITUTE AND LOCATION: NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	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.)

We wish to understand differences in metabolism and regulation between normal and cancer cells, and between cancer cells that are responsive to chemotherapy and those which exhibit multiple drug resistance (MDR). Nuclear magnetic resonance (NMR) spectroscopy is used to monitor metabolic processes noninvasively, using a perfusion technique that we have developed in which cells are embedded in agarose gel threads. With this technique we have observed with ^{31}P NMR significant differences in the levels of major phosphate metabolites in wild type (WT) MCF-7 breast cancer cells, and an adriamycin resistant cell line (Adr^R) that exhibits MDR.

One of the major differences between normal and cancer cells is the control of energy metabolism. To follow glycolysis in WT and Adr^R cells we improved the perfusion technique to enable us to use small volumes with ^{13}C-glucose. We are able to monitor the rate of glucose uptake and the concomitant rate of lactate production with ^{13}C NMR. The effects of inhibitors and drugs on glycolysis has been monitored in the perfusion system. Phospholipid metabolism in these cells can also be monitored by consideration of the levels of phosphomonoester (PME) and phosphordiester (PDE) peaks in the ^{31}P NMR spectra.

In order to extend these observations to noninvasive metabolic measurements in vivo we have designed and constructed a versatile NMR probe for our spectrometer. This contains the coils for irradiation and signal reception, and has a cradle to hold the animal. All components are adjustable and placeable, but currently are arranged to observe spectra from a subcutaneous tumor in a rodent. Eventually we anticipate extending these studies to larger animals, and hopefully to clinical trials of ^{31}P MRS of breast cancer in humans.

Differences in Phosphate Metabolite Levels in MDR: Comparison of the ^{31}P NMR spectra of perfused WT and Adr^R cells showed significant and reproducible differences in the levels of major phosphate-containing metabolites. Resistant cells demonstrated elevated levels of PCr, and depressed levels of phosphomonoesters (PME), phosphodiesters (PDEE), and diphosphodiesters (UDPG). Spectra of extracts of these cells also exhibited consistent differences in levels of these metabolites. In order to elucidate the origin of these differences experiments were carried out in two metabolic areas--energy metabolism (glycolysis) and phospholipid (PL) metabolism.

Glycolysis in Cancer Cells: Glucose utilization and lactate production have been monitored as a function of time using $^{13}C_1$-glucose with perfused wild type (WT) MCF-7 human breast cancer cells, and the drug-resistant cell line Adr^R. Compared to WT, Adr^R cells exhibited an enhanced (3X) rate of glycolysis, indicating an increased demand for ATP production. We have investigated the effects of glucose depletion and azide, an inhibitor of oxidative phosphorylation, on the levels of intracellular phosphates (Pi, ATP) and intracellular pH using ^{31}P MRS, and on the rates of glycolysis. In both cell lines, ATP levels and the rates of glucose utilization and lactate production were invariant to the presence of azide. ATP production, especially in Adr^R cells, was highly dependent on active glucose metabolism. The results of these direct measurements confirm that these cells survive by predominantly utilizing glycolysis. Glutamate and myo-inositol were observed in ^{13}C spectra of acid extracts of Adr^R but not WT cells. Both metaboltes are potential substrates in drug detoxification. These differences in rates of glycolysis, ATP production, and the production of certain metabolites may reflect metabolic adaptations associated with the development of drug resistance.

Effects of Adriamycin in Glycolysis: Oxygen radical dependent cytotoxicity has been proposed as one mechanism of action of the anticancer drug, adriamycin (ADR). Free radical formation is initiated by the reduction of ADR to its semiquinone by NADPH dependent flavin reductases. Since tumor cells typically suppress mitochondrial oxidative phosphorylation in favor of glycolysis, the hexose monophosphate shunt (HMS) is their primary source of NADPH production. Using ^{13}C magnetic resonance spectroscopy (MRS), we have demonstrated that clinically relevant concentrations (nM) of ADR stimulate the HMS activity in wild type (WT) and ADR-resistant (Adr^R) MCF-7 breast cancer cells. Glucose utilization and lactate production were monitored following the addition of 5.0 mM $^{13}C_1$-glucose during the perfusion of cells cast in agarose gel threads. The addition of 2 nM ADR reduced the production of $^{13}C_3$-lactate by WT cells from 3.4 mM to 2.6 mM and by Adr^R cells from 3.6 mM to 2.8 mM. During the conversion to pentose-5-phosphate, the C1 label of glucose-6-phosphate is exclusively oxidized to CO_2, which reduces conversion of C1 through glycolysis to the C3 position of lactate. As a control, the metabolism of 5.0 mM $^{13}C_6$-glucose was monitored, since the conversion of C6 of glucose to the C3 position of lactate is not influenced by the HMS activity. Without drug, WT cells produced 3.0 mM and Adr^R cells produced 4.4 mM $^{13}C_3$-lactate. The addition of 2 nM ADR had no effect on these levels. The difference between the two labels in the absence of drug indicates the basal HMS activity. The low level of lactate production observed for WT cells using the C6 label is unclear.

Phospholipid Metabolism in Cancer Cells: Addition of choline or ethanolamine to the perfusate of a system in which human breast cancer cells are monitored in vitro by ^{31}P NMR spectroscopy resulted in significant changes to signals attributed to phosphomonoesters (PME) and phsphodiesters (PDE). Addition of hemicholinium-3 (HC-3), an inhibitor of choline kinase, resulted in a reduction of one PME peak and an increase in the PDE peaks. These and other results enable us to directly assign the PME peaks to glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) and to define the pathways controlling their production. The PMEs arise from the activity of choline and ethanolamine kinases, the first step in phospholipid biosynthesis, and the PDEs result from the inhibition of GPC phosphodiesterase, the final step in phospholipid catabolism. Furthermore, cells at log phase growth have PC and PE peaks with twice the intensity of cells at confluency. These signals have been observed in vivo in human colon and breast tumors grown in nude mice. Since these PMEs are low in most normal (nonproliferating) tissues, their detection could form a basis for noninvasive diagnosis. Additionally, the ability to observe PE and PC, which are situated between the control enzymes of these two major synthetic pathways, will allow direct study of these pathways in intact cells and in vivo.

In Vivo Spectroscopy: A versatile in vivo NMR probe for a vertical bore magnet has been constructed. A small animal is enclosed in a sealed chamber equipped with inlet and outlet connectors for anesthetic gas. A replaceable bracket assembly positions the coil vertically to accomodate a subcutaneous tumor, which protrudes through an adjustable slot in the animal cradle. Results with three kinds of animal cradles were compared; a completely copper cradle, a plastic cradle, and a plastic cradle with a thin copper foil. The latter was found to be the best compromise for S/N, since the copper cradle resulted in line broadening, but the plastic cradle alone allowed signals from non-tumorous tissue. ^{31}P spectra of in vivo tumor tissue was similar to spectra of in vitro perfused tumor cells of the same origin, except that the tumor tissue exhibited much higher levels of inorganic phosphate and phosphocreatine. Signals from C_2-glucose and its major metabolite, C2-lactate, were readily observed and monitored in an unobstructed region of the ^{13}C spectra of tumor tissue in vivo following the injection of $^{13}C_2$-glucose in adjacent tissues. A concentric paired coil arrangement ($^{31}P/^{1}H$ or $^{13}C/^{1}H$) was found to be superior to a double tuned single coil. We have also observed ^{1}H HMR spectra of metabolites with water signal suppression. The results of these experiments are anticipated to be directly applicable to in vivo studies using the horizontal magnet spectrometers in the new NIH NMR Research Center, and also potentially to clinical studies using the whole body Signa NMR system.

PUBLICATIONS:

Cohen, J.S., Lyon, R.C., Chen, C., Faustino, P.J., Batist, G., Shoemaker, M., Rubalcaba, E., and Cowan, K.H.: Differences in Metabolite Levels in Drug Sensitive and Resistant Cell Lines by ^{31}P NMR Spectroscopy. Cancer Res. 46: 4087, 1986.

Lyon, R.C., Faustino, P.J., and Cohen, J.S.: A Perfusion Technique for ^{13}C NMR Studies of the Metabolism of ^{13}C-Labelled Substrates by Mammalian Cells. Mag. Res. Med. 3: 663, 1986.

Cohen, J.S., and Lyon, R.C.: Metabolism of Perfused Drug-Sensitive and Resistant Breast Cancer Cells. In Magnetic Resonance of the Reproductive System. (Haseltine, F., and McCarthy, S. Eds.), Slack, NY, 1987, pp. 51-66.

Lyon, R.C., Cohen, J.S., Faustino, P.J., Megnin, F. and Myers, C.E.: Glucose Metabolism in Drug-Sensitive and Drug-Resistant Human Breast Cancer Cells Monitored by Magnetic Resonance Spectroscopy. Submitted for publication.

Daley, P.F., Lyon, R.C., Faustino, P.J. and Cohen, J.S.: Phospolipid Metabolism in Cancer Cells Monitored by ^{31}P NMR Spectroscopy. Submitted for publication.

Cohen, J.S. and Lyon, R.C.: Multinuclear NMR Study of the Metabolism of Drug-Sensitive and Drug-Resistant Human Breast Cancer Cells. NY Acad. Sci., in press.

Lyon, R.C., Tschudin, R.G., Daly, P.F. and Cohen, J.S.: A Versatile Multinuclear Probe Designed for In Vivo Spectroscopy: Applications to Subcutaneous Human Tumors in Mice. Submitted for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06520-04 CP

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Magnetic Resonance Imaging of Tumors: Contrast Agents

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

Jack S. Cohen, Ph.D.	Research Chemist	CPB, COP, DCT, NCI
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Arthur Katz, Ph.D.	Guest Researcher	CPB, COP, DCT, NCI
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Frederique Megnin, M.Sc.	Guest Researcher	CPB, COP, DCT, NCI

COOPERATING UNITS (if any)

Inst. for Diagnostic Research, Berlin, West Germany

LAB/BRANCH: Clinical Pharmacology Branch

SECTION: Biophysical Pharmacology Branch

INSTITUTE AND LOCATION: NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Magnetic resonance imaging (MRI) is an important tool in diagnostic radiology, and allows discrimination between a growth and surrounding soft tissue. The contrast depends on the relaxation times (T_1 and T_2) of the bulk water in the respective tissues. A paramagnetic substance is one that contains an unpaired electron and alters the relaxation rate of water. Our objective is to identify a class of paramagnetic contrast agents which are selectively retained by tumors, in order to facilitate and clarify MRI tumor contrast.

We have chosen the water-soluble metalloporphyrins (WSMPs) for several reasons; porphyrins are known to be selectively retained by tumors, they form complexes with many metal ions, and the synthetic water soluble porphyrins are much more tractable than the hydrophobic natural heme products. We have evaluated several of these WSMPs by several criteria: solubility; relaxivity; stability in human plasma and in vivo; distribution in vivo; toxicity; and MRI contrast properties. As a result of these comparisons we have identified Mn(III)TPPS as a potential tumor contrast agent in MRI. We are currently studying the mechanism of tumor retention of porphyrins and WSMPs.

MnTPPS as an MRI Contrast Agent for Tumors:

We had previously reported that Mn(III) complexes with porphyrins exhibit more efficient relaxivity than the corresponding Fe(III) complexes, and than the MRI contrast agent GdDTPA. We concluded that MnTPPS is a potential contrast agent for tumors in MRI. A similar study is underway on Mn(III) uroporphyrin I complex, that has suitable properties of relaxivity and solubility. Using the in vivo NMR imagers now available in the new NIH NMR Center we are planning a comparative study of dose/contrast relationship for a series of contrast agents and tumor models.

Mechanism of Uptake Stability:

We are attempting to elucidate the mechanism of porphyrin/MP uptake by tumor cells. Initially we compared results with MCF-7 breast cancer cells, both wild type and an adriamycin resistant cell line (Adr^R). This is known to contain 45x the level of glutathione S-transferase, which is known to avidly bind porphyrins and other xenobiotics. In a perfused in vitro system no significant difference was observed in the relaxation rate upon uptake of MnTPPS in both cell lines. Also the rate of wash-out after 3 hours was approximately the same as the rate of uptake, and there was little residual retention. This would indicate that protein binding to GST is not the mechanism of porphyrin/MP retention in tumor cells, and that no significant chemical or enzymatic modification or compartmentalization occurs within a period of hours.

To quantitate MnTPPS stability we are determining the fluorescence in cells incubated in MnTPPS, since the intact MP is not fluorescent, unlike the free porphyrin. Detailed in vivo distribution stability studies, are underway using atomic absorption of Mn. The object is to decide if MnTPPS can be considered for clinical trials.

PUBLICATIONS:

Lyon, R. C., Faustino, P. J., Cohen, J. S. et al.: Tissue Distribution and Stability of Metalloporphyrin MRI Contrast Agents, Magn. Reson. Med. 4: 24, 1987,

Cohen, J.S., and Lyon, R.: Metalloporphyrins as Potential Contrast Agents in MRI, in Contrast Agents in MRI, (Excerpta Medica), 1986, p. 35.

Megnin, F., Faustino, P., Lyon, R. and Cohen, J.S.: On the Mechanism of Porphyrin and Metalloporphyrin Uptake in Cancer Cells. Biochim. Biophys. Acta., in press.

Hambright, P., Turner, A., Cohen, J.S., Lyon, R.C., Katz, A., Neta, P. and Adeyemo, A.: An Iron (III) Porphyrin that Exhibits Minimal Dimerization in Aqueous Solution. Inorg. Chim. Acta. 128: L11, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06521 04 CP

PERIOD COVERED: October 1, 1986 to September 30, 1987

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

Conformations and Interactions of Nucleic Acids, Proteins and Drugs in Solution

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

Jack S. Cohen, Ph.D.	Research Chemist,	CPB, COP, DCT, NCI
Babul Borah, Ph.D.	Vis. Assoc.	CPB, COP, DCT, NCI
Charles E. Myers, M.D.	Chief	CPB, COP, DCT, NCI
Siddhartha Roy, Ph.D.	Vis. Assoc.	CPB, COP, DCT, NCI
Patrick Faustino, M.Sc.	Chemist	CPB, COP, DCT, NCI
Peter Daly, M.D.	Clin. Assoc.	POB, COP, DCT, NCI

COOPERATING UNITS *(if any)*

Dennis A. Torchia, NIDR; Gerald Zon, BB/FDA; Ettore Appella, DCBD/NCI; Heisaburo Shindo, Tokyo College of Pharmacy, Tokyo, Japan; Maria Miller, NBS; Todd Miles, LMB/NIADDK; Joel Sussman, Weizmann Inst., Israel

LAB/BRANCH: Clinical Pharmacology Branch

SECTION: Biophysical Pharmacology Section

INSTITUTE AND LOCATION: NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

We wish to eluciate the detailed conformations of DNA in solution, and to investigate their relationships to genetic function, interactions with drugs, and protein recognition. The method we have chosen to carry out such studies in solution is nuclear magnetic resonance (NMR) spectroscopy.

We have tested the factors which contribute to the B to Z conformational transition in polydeoxynucleotides, and we have studied the solution conformation of the homopolymer poly(dA).poly(dT) about which there has been controversy in the literature. The specific method used to detail these solution conformations is the 2D-NOE NMR method, which provides relative interatomic distances.

Loops in DNA are expected to be important for protein recognition of selective genetic sites, such as RNA polymerase promotor sequences. Consequently, we have studied quasipalindromic synthetic oligonucleotides, and have delineated the mechanism of hairpin loop formation.

In order to specify the mobility of different portions of the DNA molecule, particularly the deoxyribose ring, we have carried out novel syntheses of selectively deuterated nucleosides. This allows us to use solid state deuterium NMR, which is the least ambiguous NMR method available to determine local molecular mobility.

B to Z or A-Form Transition: We have recently shown that the synthetic copolymer duplex poly(d2NH_2A-dT) exists in an A-form conformation in high salt. This was indicated by the pattern of cross-peaks in 2D-NOE spectra, which were quite distinct from those found for B or Z-form DNA. Previous work had assumed a Z form based on the CD spectra of the high salt form, and the fact that poly-d GC gives a Z form, while poly-d AT does not. In order to extend and generalize these observations we obtained the 2D-NOE spectra of poly (d2NH_2A-d5XU) at different salt concentrations (where X=Br, I). In both cases a similar pattern of cross peaks with 3' proton interactions was observed, consistent with an A-form in high salt in these cases also. These observations represent the first examples of an A-form of DNA in high salt solution, and also indicate that neither 2-amino-purine nor 5-halogenopyrimidine substitution are sufficient to produce a Z-form.

Conformation of Homopolydeoxynucleotide: It has recently been proposed that the homopolymer poly(dA).poly(dT) exists in a heteronomous conformation based on x-ray diffraction results, with the A-chain in an A-form and the T-chain in a B form. Results of 2D-NOE spectra of both the homopolymer and the model oligonucleotide $d(A_6T_6)_2$ show that this is not the case, and that these sequences exist in a B conformation.

Loop Formations: Conformational NMR analysis and thermodynamic measurements have been performed on a series of oligodeoxynucleotides related to the dodecamer sequence d(CGCGAATTCGCG) which has been shown to be a B-form by Dickerson et. al. The oligomer d(CGCGAATTACGCG) was studied for hairpin loop formation in solution since it has an extra non-complementary A at position 9. It was found that this sequence exists as a stable hairpin loop in solution at room temperature. From the thermodynamics of the loop-duplex equilibrium, and the fact that the 17mer with extra CG base pairs at each end exhibits a much slower equilibrium, it was concluded that the transition does not involve a cruciform structure with branch migration, but rather requires complete strand separation for loop formation. By contrast, the extended sequence d(CGCGAAATTTACGCG) was found to prefer duplex to loop formation, since it has two extra central base pairs. From interbase NOE measurements it was shown that this sequence exists as a bent or S-shaped conformation.

PUBLICATIONS

Borah, B., Howard, F.B., Miles, H.T., and Cohen, J.S.: Conversions of poly(d2NH_2A-d5XU) from B to A Forms in High Salt. An NMR and CD Study. Biochemistry, 25: 7464, 1986.

Shindo, H., Hiyama, Y., Roy, S., Cohen, J.S., and Torchia, D.A.: Deuterium NMR of Oriented DNA Fibers. Bull. Chem Soc. Japan 60: 1631, 1987.

Roy, S., Borah, B., Zon, G., and Cohen, J.S.: Conformation of d(A_6T_6) by 2D-NOESY and its Relevance to Poly(dA).Poly(dT). Biopolymers, 26: 525, 1987

Roy, S., Weinstein, S., Borah, B., Nickol, J., Appella, E., Sussman, J., Miller, M., Shindo, H., and Cohen, J.S.: The Mechanism of Oligonucleotide Loop Formation in Solution. Biochemistry, 25: 7417, 1986.

Cohen, J.S.: 2D-NOESY of DNA: As Easy as A, B, Z. Trends in Biochemical Sciences, 12: 133, 1987.

Roy, S., Skenar, V., Appella, E., and Cohen, J.S.: Conformational Perturbation Due to an Extra Adenosine in a Self-Complementary Oligodeoxynucleotide Duplex. Biopolymers, in press.

Miller, M., Kirchhoff, W., Schwartz, F., Appella, E., Chieu, Y.H., Cohen, J.S., and Sussman, J.L.: Conformation Transitions of Synthetic DNA Sequences with Inserted Bases Related to the Dodecamer d(CGCGAATTCGCG). Nucl. Acids. Res. 15: 3877, 1987

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06523 03 CP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Metabolism, Irreversible Binding and Mechanism of Action of Etoposide (VP-16,213) to Cellular Macromolecules

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

Birandra K. Sinha, Ph.D.	Pharmacologist	CPB, COP, DCT, NCI
Charles E. Myers, M.D.	Chief	CPB, COP, DCT, NCI
Nissim Haim, M.D.	Guest Researcher	CPB, COP, DCT, NCI

COOPERATING UNITS *(if any)* Dr. J. Nemec, Frederick Cancer Center; Dr. K. Kennedy, Dept. of Pharmacology, George Washington University, Wash. D.C.; Dr. B. Kalyanaraman, Medical College of Wisconsin, Miwaukee, WI.

LAB/BRANCH

Clinical Pharmacology Branch

SECTION

Biochemical Pharmacology Section

INSTITUTE AND LOCATION

NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 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.)*

VP-1-16 undergoes O-demethylation to generate active intermediates that binds to protein and DNA. The O-demethylation is P450 dependant. Peroxidases, such as horseradish or prostaglandin synthetase, also activate VP-16 and VM-26 to their O-Quinonderivatives, and catalyze binding of reactive inermediates to DNA. Enzymatic activation to reactive intermediates may be important in the biological activities of VP-16 and VM-26.

The semisynthetic podopyllotoxin derivative, etoposide (VP-16) has shown activity against a number of human tumors. Although the mechanism of action of this drug is not clear, DNA damage induced by VP-16 has been suggested for its cytotoxicity. Recently, we have proposed that the cellular damage induced by VP-16 may result from the formation of a reactive intermediate during bioactivation of the drug. We have studied the metabolism of VP-16 by mouse hepatic microsomes. Using HPLC analysis of the chloroform extracts of the microsomal incubation it was shown that VP-16 formed the 3'-4' dihydroxyl derivative (DHVP). The formation of this metabolite (2% of the parent drug) was NADPH- protein-VP-16-and time-dependant suggesting that the activation was enzymatic. Moreover, DHVP formation was inhibited by SKF-525A and piperonylbutoxide suggesting that the 0-demethylation was P-450 dependant. Incubation of [^{3}H] VP-16 with microsomes containg NADPH and DNA resulted in irreversible binding of the drug to DNA and proteins.

Recently, we have found that peroxidase catalyzed activation of VP-16 forms a number of reactive metabolites. HPLC and mass spectral analysis have shown that VP-16 undergoes aoasfjzation (to Ar-VP-16) which is subsequently 0-demethylated to 0-Quinine (Ar-VP-16-Q). Inhibion studies suggest that the protein binding species result from 0-demothylation reactions (VP-16-Q and Ar-VP-16-Q) and that DNA binding species are positively charged.

Using alkaline elution studies in a sensitive and resistant MCF-7 cells recently, we have found that VP-16 induces significant amount of DNA damage in the sensitive cells. In contast, very little DNA damage could be detected in the resistant cells. Furthermore, when isolated nuclei were used to assess DNA damage, there was only two-fold difference in VP-16 induced DNA strand breaks between the sensitive and resistant cells. The differences in toxicity (~200 fold), and uptake of VP-16 (2-3- fold) do not completely explain DNA damage induced by VP-16 in these cells and suggest that other factors may also be involved in the mechanics of cell kill by VP-16.

PUBLICATIONS:

Sinha, B.K. and Myers, C.E.: Irreversible Binding of Etoposide (VP-16, 213) to Deoxyribonucleic Acid and Proteins. Biochem. Pharmacol. 22: 3725-3728, 1984.

Sinha, B.K., Trush, M.A., and Kalyanaraman, B.: Microsomal Interactions and Inhibition of Lipid Peroxidation by Etoposide (VP-16, 213): Implication for Mode of Action. Biochem. Pharmacol. 34: 2036-2040, 1985.

Haim, N., Roman, J., Nemec, J., and Sinha, B.K.: Peroxidative free radical famation and O-demethylation of etoposide and teniposide. Biochem. Biophys. Res. Commun. 135: 215-220, 1986.

Haim, N., Nemec, J., Roman, J., and Sinha, B.K.: In Vitro Metabolism of Etoposide (VP-16213) by Liver Microsomes and Irreversible Binding of Reactive Intermediates to Microsomal Proteins. Biochem. Pharmacol. 36: 527-536, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06524 01 CP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Inhibition of Gene Expression by Oligodeoxynucleotide Analogs

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

Jack S. Cohen, Ph.D.	Research Chemist	CPB, COP, DCT, NCI
Kazuo Shinozuka, Ph.D.	Visiting Associate	CPB, COP, DCT, NCI
Cy Stein, M.D., Ph.D.	Clinical Associate	MOB, COP, DCT, NCI
Christine Subasinghe, B.S.	NCI Fellow	CPB, COP, DCT, NCI

COOPERATING UNITS *(if any)*

S. Broder, M. Matsukura, and H. Mitsuya, COP/DCT; G. Zon, Applied Biosystems Inc., Foster City, CA; L. Neckers, LP/DCBD

LAB/BRANCH

Clinical Pharmacology Branch

SECTION

Biophysical Pharmacology Section

INSTITUTE AND LOCATION

NIH, National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Normal oligodeoxynuceotides (ODNs) have been reported to have inhibiting effects on selected gene expression, primarily due to transcription arrest by mRNA duplex formation. However, these compounds are susceptible to digestion by cell nucleases. Nuclease-resistant ODN analogs containing methylphosphonate ($P\text{-}CH_3$) in place of phosphate has been developed to overcome this problem, but these compounds also have disadvantages, notably poor aqueous solubility and poor hybridizability. In order to overcome these difficulties, we have synthesized a series of phosphorothioate (P-S) analogs, which retain the charge of phosphate, but are nuclease-resistant. These compounds are being tested as agents against HIV and other lentiviruses, against Herpes Simplex Virus (a DNA virus), and several oncogenes (eg. myc) in lung cancer cells, and against genes related to drug resistance (eg. P170 genes). Tests for toxicity in mice are also being conducted. A series of analogs with covalently attached groups on both the 3' and 5' ends of oligomers have been synthesized and will also be tested for biological activity in a cell free expression system and in the systems tested above if further evidence of improved activity is discovered.

MAJOR FINDINGS:

Nuclease-resistant phosphorothioate analogs of certain oligodeoxynucleotides have been tested in vitro as antiviral agents against human immunodeficiency virus (HIV) in a human T-cell (ATH8) assay. Phosphorothioate analogs complementary to HIV sequences, as well as non-complementary analogs including homo-oligomers, exhibited potent antiviral activity. The antiviral activity was related to the base composition of the analogs, and longer phosphorothioates were more effective than shorter ones. A 28-mer phosphorothioate oligodeoxycytidine (S-dC_{28}) at a concentration of 1 uM exhibited potent antiviral activity and inhibited de novo viral DNA synthesis as shown by Southern blot analysis. However, an anti-sense phosphorothioate 28-mer complementary to art/trs, as well as the homo-oligomer S-dC_{28}, failed to inhibit gag expression in chronically infected T-cells assessed by immunofluorescent assay at concentrations up to 25uM. An N^3-methylthymidine-containing phosphorothioate analog, which does not hybridize efficiently in vitro to complementary normal DNA, showed no antiviral activity. The phosphorothioate analog S-dC_{14} synergistically enhanced the antiviral activity of 2', 3'-dideoxyadenosine. Therefore, phosphorothioate analogs of oligodeoxynucleotides could represent a novel class of experimental therapeutic agents against the acquired immunodeficiency syndrome (AIDS) and related diseases. Preliminary results of inhibition of other genes (myc in HL60 cells) are encouraging.

The intermediates of S-ODNs with attached linkers at both 3' and 5' ends have been synthesized, to which any chosen terminal group can be added. Several initial compounds with intercalator groups attached have been synthesized and are now being purified and characterized.

PUPLICATIONS:

Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J.S., and Broder, S.: Phosphorothioate Analogs of Oligodeoxynucleotides: Novel Inhibitors of Replication and Cytopathic Effects of Human Immunodeficiency Virus (HIV), Proc. Natl. Acad. Sci. USA, in press.

Stein, C., and Cohen, J.S.: Towards a New Chemotherapy, submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 03403-22 M

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Clinical Trials and Miscellaneous Clinical Investigations

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

PI:	Robert C. Young	Chief	M	NCI
Other:	Charles Myers	Chief	CP	NCI
	Marc Lippman	Sr Investigator	M	NCI
	Edward Gelmann	Sr Investigator	M	NCI
	Dan Longo	Assoc Director	BRMP	NCI
	Louis Matis	Sr Staff Fellow	M	NCI
	Neal Rosen	Sr Investigator	M	NCI

COOPERATING UNITS *(if any)*

Radiation Oncology Branch, NCI; Navy-MOB, NCI; Clinical Pharmacology Branch NCI; Biometric Research Branch, NCI; Surgery Branch,NCI; Immunology Branch, NCI; Biological Response Modifiers Program, NCI; Environmental Epidemiology Branch, NCI.

LAB/BRANCH

Medicine Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
31	22.5	8.5

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 Medicine Branch is a major clinical facility of the NCI. Its activities are divided between clinical therapeutic trials in cancer patients and related laboratory research. Clinical trials of cancer treatment are currently underway in breast cancer, ovarian cancer, Hodgkin's disease, non-Hodgkin's lymphomas, testicular tumors, Kaposi's sarcoma, AIDS, soft tissue sarcomas, prostate cancer, pheochromocytoma and melanoma. Phase I-II clinical trials have been completed this year on the following new experimental agents: TCNP and CBDCA. Phase I-II trials continue on TNF + γ-interferon, dideoxycytidine, diethyldithiocarbamate, I.P. LAK and IL-2, and intraperitoneal chemotherapy of aclacinomycin. In 1986-1987 the Medicine Branch staff published or has accepted for publication 140 papers, articles, or book chapters. This is one of the largest number of scientific publications in the history of the Branch. Forty-one active protocols are maintained primarily by the Medicine Branch and over 1500 patients patients are on clinical trials, 1412 (94%) at the Medicine Branch, 72 (5%) at the Navy-MOB and 16 (1%) at the University of Maryland. Details of the clinical and laboratory studies will be reviewed in the subsequent sections. Additional summaries of clinical studies are summarized under reports entitled, Clinical Program in Breast Carcinoma. Laboratory research of the Branch is summarized under reports entitled, Mechanisms of Drug Resistance, Multidrug Resistance and Cisplatin Resistance, Cytogenetic Studies, Adoptive Cellular Immunotherapy of Cancer, Mechanisms of Hormone Dependence of Human Malignancy, Transforming Genes and Determinants of Hormone-Independence in Hormone Responsive Human Malignancies, and Genetic Control of Human Colon and Breast Cancer.

Robert Ozols	Sr Investigator	M	NCI
Antonio J. Fojo	Senior Investigator	M	NCI
Michael Bookman	Senior Staff Fellow	M	NCI
Jacqueline Whang-Peng	Sr Investigator	M	NCI
Vincent T. DeVita, Jr.	Director		NCI
Samuel Broder	Associate Director	COP	NCI
Seth Steinberg	Head	BR	NCI
Steven Rosenberg	Chief	S	NCI
Eli Glatstein	Chief	RO	NCI
Daniel Ihde	Sr Investigator	Navy-MOB	NCI
Carl Freter	Senior Staff Fellow	M	NCI
Ron Steis	Senior Investigator	BRMP	NCI
Elaine Jaffe	Sr Investigator	LP	NCI
Gregory Curt	Sr Investigator	OD,DCT	NCI
Susan Hubbard	Chief	SI	NCI
Ami Ostchega	Chemo Res Nurse	M	NCI
Joan Jacob	Chemo Res Nurse	M	NCI
Elizabeth Egan	Chemo Res Nurse	M	NCI
Pat Duffey	Chemo Res Nurse	M	NCI
First and Second Year Medical Staff Fellows		M	NCI

Major Accomplishments in 1986-1987

Hodgkin's Disease: Established

1) Early Hodgkin's disease: Radiation therapy vs. combination chemotherapy: While radiation therapy is generally successful in the management of early stage Hodgkin's disease, as many as 25% of patients relapse from radiation-induced complete remissions and, although many can be salvaged by chemotherapy, this is accomplished at some risk of induced second malignancy. Because combination chemotherapy is curative in advanced disease and can salvage many patients who relapse after radiation therapy and because small trials with MOPP chemotherapy in early stage Hodgkin's disease appeared successful, the Medicine Branch and Radiation Oncology Branch are performing a randomized comparison between these treatments. Thus far, 46 patients have been randomized to chemotherapy and 44 to radiation therapy. The complete remission rates are 100% for combination chemotherapy, and 95% for radiation therapy. With a median follow-up in excess of 40 months, 9% of the MOPP treated patients have relapsed compared to 36% for those treated with radiation therapy. Disease-free survival in randomized patients is significantly different ($p_2 = .004$) in favor of the MOPP chemotherapy, but overall survival is not ($p_2 = 0.089$). Initial results of this trial establish uniformly high complete remission rates with both modalities and better disease-free survival for MOPP chemotherapy. If further follow-up substantiates these observations, this trial will establish MOPP chemotherapy as an excellent alternative to radiation therapy in the management of early Hodgkin's disease.

2) Massive Mediastinal Hodgkin's Disease: Massive mediastinal Hodgkin's disease represents one of the most difficult remaining problems in Hodgkin's disease management. Combined modality approaches appear to be the most successful in this group and we are studying MOPP-ABVD followed by aggres-

sive simulator-designed radiation therapy for patients with all stages of Hodgkin's disease who have massive mediastinal involvement. Fifty-three patients have been entered on study. Eighty-two percent of patients have entered a complete remission and only six of the complete remissions have relapsed (14%) with a median duration in excess of 27+ months. Overall survival and disease-free survival at four years exceeds 80%. This result in all stages of patients with massive mediastinal disease compares very favorably with the approximately 50-60% long-term disease-free survival previously reported for patients with Stage I & II massive mediastinal disease.

3) Advanced Hodgkin's disease: MOPP vs. alternating non-crossresistant combinations (MOPP-CABS): A randomized trial of MOPP vs. MOPP-CABS in advanced Hodgkin's disease is one of the few trials of an alternating non-cross-resistant combination other than MOPP-ABVD. One hundred and twenty-seven patients have been randomized. Initial complete remission rates are 92% for MOPP and 88% for MOPP-CABS. With a median follow-up in excess of four years, 75% of both groups of patients remain alive and there is no significant difference between the two arms at this point. This alternating sequence regimen does not appear to produce better results than MOPP alone, although the results in both arms are as good as in the Bonadonna trial of MOPP-ABVD. Replacement protocols will test dose-intensified induction with MOPP with GM-CSF to facilitate marrow rescue.

Hodgkin's Disease: Published

1. Dmitrovsky, E., Martin, S.E., Krudy, A.G., Chu, E.W., Jaffe, E.S., Longo, D.L., and Young, R.C.: Lymph node aspiration in the management of Hodgkin's disease. J. Clin. Oncol. 4: 306-310, 1986.

2. Longo, D.L., Young, R.C., Wesley, M., Hubbard, S.M., Duffey, P.L., Jaffe, E.S., and DeVita, V.T.: Twenty years of MOPP therapy for Hodgkin's disease. J. Clin. Oncol. 4: 1295-1306, 1986.

3. Blayney, D.W., Longo, D.L., Young, R.C., Greene, M.H., Hubbard, S.M., Postal, M.G., Duffey, P.L., DeVita, V.T.: Decreasing leukemia risk with prolonged follow-up after chemotherapy and radiotherapy for Hodgkin's disease. N. Engl. J. Med. 316: 710-714, 1987.

4. Kant, J.A., Hubbard, S.M., Longo, D.L., Simon, R.M., DeVita Jr., V.T., and Jaffe, E.S.: The pathologic and clinical heterogeneity of lymphocyte-depleted Hodgkin's disease. J. Clin. Oncol. 4: 284-294, 1986.

5. Bookman, M.A. and Longo, D.L.: Concomitant illness in patients treated for Hodgkin's disease. Cancer Treat. Rev. 13: 77-111, 1986.

6. Edington, H., Salwitz, J., Longo, D.L., Roth, J.A., and Pass, H.: Thymic hyperplasia masquerading as recurrent Hodgkin's disease: Case report and review of the literature. J. Surg. Oncol. 33: 120-123, 1986.

7. Simrell, C.R., Boccia, R.V., Longo, D.L., and Jaffe, E.S.: Coexisting Hodgkin's disease and mycosis fungoides: Immunohistochemical proof of its existence. Arch. Pathol. Lab. Med. 110: 1029-1034, 1986.

8. Longo, D.L.: Future prospects for management of patients with Hodgkin's disease. In Future Prospects for Cancer Therapy, Magrath, I. (Ed.), Geneva, UICC/Springer Publishers, 1987 (in press).

Non-Hodgkin's Lymphoma: Established:

1) Stage I Diffuse Aggressive Lymphoma: Forty-three patients with clinically staged early (Stage I) diffuse aggressive lymphoma have been treated with a modified ProMACE-MOPP regimen at 75% doses on a monthly basis for 4 months followed by involved field radiation therapy. This treatment is given entirely in the outpatient clinic. There have been no treatment related deaths, few hospitalizations for leukopenia and 41/43 (95%) of patients have entered a complete remission. To date none of the complete remissions has relapsed. This regimen appears to eliminate the necessity for surgical staging in these early disease patients and is an outpatient regimen with modest toxicity producing a complete remission rate of 95%. At 4 years follow-up the overall survival is 95%.

2) Indolent Lymphoma Trial: One hundred-and-four patients with advanced stage favorable prognosis non-Hodgkin's lymphomas have been randomized to receive either "watch and wait" therapy or intensive chemotherapy with ProMACE-MOPP and total nodal irradiation. Of those randomized to aggressive combination chemotherapy and evaluable, 76% of patients have entered a complete remission and median duration of initial remission will exceed 50+ months. Eighty-four percent of patients who achieved complete remissions are still in their first remission. Of those patients randomized to "watch and wait", 57% remain off therapy although 39% have received limited radiation treatment. Forty-two percent of patients have been crossed over to the intensive treatment arm with a median time to crossover of 34 months and, of those, 43% have entered a complete remission with intensive treatment. Of interest, 7 patients have undergone histologic progression without exposure to any chemotherapy. Several preliminary conclusions can already be derived from this study. (1) Approximately half of patients with minimal therapy will remain relatively asymptomatic for periods of time in excess of two years. (2) Initial aggressive chemotherapy and radiation treatment will produce a high complete remission rate and appears to have reduced the incidence of relapse after complete remission, and changed the natural history of lymphoma. (3) Histologic progression occurs in some patients without exposure to cytotoxic therapy and is therefore a part of the intrinsic natural history of disease.

3) Advanced Diffuse Aggressive Lymphomas: ProMACE-MOPP vs. ProMACE-CytaBOM: One hundred and forty-eight patients with advanced diffuse aggressive lymphoma have been treated with either ProMACE-MOPP or ProMACE-CytaBOM. Complete remissions have been achieved in 57/75 (76%) of those treated with ProMACE-MOPP and 58/73 (79%) of those with ProMACE-CytaBOM. Median follow-up of the complete remissions now exceeds 30 months and 32% of patients on the

ProMACE-MOPP arm have relapsed compared to 24% on ProMACE-CytaBOM. Only 30% of all patients on study have died. There were only five granulocytopenic deaths (4%), and there have been none on ProMACE-CytaBOM since the institution of bactrin prophylaxis. Both the modified ProMACE-MOPP regimen and the ProMACE-CytaBOM have very high complete remission rates and both have less granulocytopenic deaths than ProMACE-MOPP flexitherapy. Both of these new regimens have reduced the incidence of toxic deaths (4% vs. 10%) compared to the previous ProMACE-MOPP flexitherapy regimen, are comparable in efficacy, and are given in the outpatient clinic.

Non-Hodgkin's Lymphoma: Published

1. Longo, D.L. and Broder, S.: Lymphoproliferative diseases. In Samter, M. (Ed.): Immunological Diseases, 4th Edition. Boston, Little, Brown, and Company, 1987 (in press).

2. Longo, D.L. and DeVita, V.T., Jr.: Malignant Lymphomas. In Pinedo, H.M. and Chabner, B.A. (Eds.): In Cancer Chemotherapy Annual 8, Amsterdam, Elsevier Science Publishers, 1986, pp. 262-304.

3. Matis, L.A., Young, R.C., and Longo, D.L.: Nodular lymphomas - Current Concepts. CRC Crit. Rev. Oncol/Hematol. 5: 171-197, 1986.

4. Fisher, R.I., DeVita, V.T., Longo, D.L. and Young, R.C.: Treatment of diffuse aggressive lymphoma with "ProMACE" based combination chemotherapy. In Skarin, A.T. (Ed.): Update on Treatment for Diffuse Large Cell Lymphoma, New York, Park Row Publishers, 1986, pp. 31-35.

5. DeVita, V.T., Longo, D.L., Hubbard, S.M., Young, R.C., and Chabner, B.A.: The Lymphomas: Biologic implications of therapy and therapeutic implications of the new biology. In Malignant Lymphoma, Berard, C.W., Dorfman, R.F., and Kaufman, N. (Eds.), Williams & Wilkins, 1987, pp. 249-267.

6. Browne, M.J., Hubbard, S.M., Longo, D.L., Fisher, R., Wesley, R., Ihde, D.C., Young, R.C., DeVita, V.T., and Pizzo, P.A.: Excess prevalence of pneumocystis carinii pneumonia in lymphoma patients treated with combination chemotherapy. Ann. Intern. Med. 104: 338-344, 1986.

7. Young, R.C., Fisher, R.I., and Longo, D.L.: Non-Hodgkin's Disease: Unfavorable Lymphomas. In Current Therapy in Internal Medicine, Bayless, Brain, & Cherniack (Eds.), B.C. Decker, Inc., 1987, pp. 386-390.

8. Little, C. and Longo, D.L.: Non-Hodgkin's lymphomas: Epidemiology, etiology, pathology, natural history, and disease evolution. In Moossa, A.R., Robson, M.C., and Schimpff, S.C. (Eds.): Comprehensive Textbook of Oncology, Baltimore, Williams and Wilkins, 1986, pp. 574-580.

9. Little, C. and Longo, D.L.: Non-Hodgkin's lymphoma: Diagnosis and management. In Moossa, A.R., Robson, M.C., and Schimpff, S.C. (Eds.): Comprehensive Textbook of Oncology, Baltimore, Williams and Wilkins, 1986, pp. 581-592.

10. Foon, K.A., Nakano, G.M., Koller, C.A., Longo, D.L., and Steis, R.G.: Response to 2'Deoxycoformycin after failure of interferon-alpha in non-splenectomized patients with hairy cell leukemia. Blood 68: 297-300, 1986.

11. Longo, D.L., DeVita Jr., V.T., and Hubbard, S.M.: Non-Hodgkin's lymphomas. In Slevin, M.L. and Staquet, M.J. (Eds.): Randomized Trials in Cancer: A Critical Review by Sites. New York, Raven Press, 1986, pp. 91-130.

12. Longo, D.L. and Devita Jr., V.T: Malignant lymphomas. In Pinedo, H.M., and Chabner, B.A. (Eds.): Cancer Chemotherapy/8. Amsterdam, Elsevier Science Publishers B.V., 1986, pp. 262-304.

13. Urba, W.J. and Longo, D.L.: Alpha interferon in the treatment of nodular lymphomas. Semin. Oncol. 13: 40-47, 1986

14. Steis, R.G., Foon, K.A., and Longo, D.L.: Current and future uses of recombinant alpha-interferon in the treatment of low-grade non-Hodgkin's lymphoma. Cancer 59: 658-663, 1987.

15. Raffeld, M., Wright, J.J., Lipford, E., Cossman, J., Longo, D.L., Bakhshi, A., and Korsmeyer, S.J.: Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. Cancer Res. 47: 2537-2542, 1987.

16. Longo, D.L. and Broder, S.: Lymphoproliferative diseases. In Samter, M. (Ed.): Immunological Diseases, 4th Edition. Boston, Little Brown and Co., 1987 (in press).

17. Urba, W.J. and Longo, D.L.: Diffuse aggressive lymphoma in adults. In Magrath, I. (Ed.): Future Prospects for Cancer Therapy. Geneva, UICC/ Springer Publishers, 1987 (in press).

18. Groves, E. and Longo, D.L.: Progression of lymphoproliferative disorders and hematologic malignancies. In Kaiser, H.E. (Ed.): Progressive Stages of Malignant Neoplastic Growth. The Netherlands, Martinus Nijhoff Publishers, 1987 (in press).

19. Longo, D.L. and Hathorn, J.: Current therapy of diffuse large cell lymphoma. In Brown, E.B. (Ed.): Progress of Hematology, Volume XV. Orlando, Grune & Stratton, Inc., 1987 (in press).

20. Urba, W.J. and Longo, D.L.: Cutaneous T-cell lymphoma. In Brain, M.C. and Carbone, P.P. (Eds.): Current Therapy in Hematology/Oncology-3. Toronto, B.C. Decker Inc., 1987 (in press).

Ovarian Carcinoma: Established:

1) Early Ovarian Cancer: Two hundred and twenty-nine patients have now been randomized to two separate clinical trials in early ovarian cancer and the studies are now closed for new patient entry. The first includes 92 patients with Stage IA and IB disease and compares adjuvant melphalan to

no additional therapy after comprehensive surgical staging. The second trial includes 137 patients with minimal residual disease and compares melphalan to intraperitoneal P^{32}. This clinical trial performed in conjunction with the Ovarian Cancer Study Group and GOG is the only randomized trial in early ovarian cancer treatment in the United States. Median follow-up now exceeds 5 years for both trials and the following conclusions are established. 1) carefully staged patients with Stage IA_1 and IB_1 disease with well- or moderately well-differentiated histology have an extremely good prognosis (5 year survival exceeds 94% even if no adjuvant therapy is given; 2) other patients with Stage IA-IIC disease, even after careful surgical staging experience a 20% recurrence and 12% death rate in the first two years after surgery but overall survival is still very good (5 yr survival >79%) regardless of whether I.P. radioisotopes or chemotherapy is administered. This compares very favorably with historical 5-year survivals of approximately 50-60% for similar groups of conventionally staged patients. Such patients are appropriate for adjuvant therapy. A replacement protocol comparing P^{32} to a short course (3 months) dose-intense combination chemotherapy with cyclophosphamide-cisplatin has been initiated.

2) Diethyldithiocarbamate (DDTC) as a Protector from Cisplatin Toxicity. Clinical studies have demonstrated that high dose cisplatin and high dose carboplatin are very active in ovarian cancer, but are limited by peripheral neuropathy or severe myelosuppression, respectively. Recent experimental studies have demonstrated that diethyldithiocarbamate (DDTC) can protect against the nephrotoxicity of cisplatin and the myelosuppression of carboplatin without reducing the antitumor effect. DDTC is one of a family of sulfur nucleophiles capable of reducing platinum toxicity. It has been used clinically for nickel-carbonyl poisoning and in Wilson's disease. It gets into brain and into peripheral nerves (unlike WR2721). In studies in man, it has been without significant long term toxicity. It is unique in that its protective effect is seen even if it is administered 1/2 to 4 hours after cisplatin administration. Preliminary evidence from S^{35} labelled studies suggests that the drug may be preferentially distributed to normal tissues rather than tumor and its protective effect may relate to pharmacokinetic distribution differences. Thus far, we have treated 9 patients with refractory ovarian cancer with high dose CBDCA (800 mg/M^2 I.V. day 1) followed by DDTC 4 g/M^2 I.V. 3 hours after CBDCA. Preliminary evidence is that both thrombocytopenia and leukopenia are significantly reduced and that antitumor effect is not compromised.

3) CPR for Advanced Ovarian Cancer: Cyclophosphamide, high dose cisplatin and total abdominal irradiation are now being used in advanced previously untreated patients. Fifty-two patients have been treated. After only 3-4 months of induction chemotherapy, clinical reevaluation was undertaken and patients clinically free of disease were restaged. Sixty-five percent of patients achieved a clinical complete remission and 56% of those have been pathologically free of disease at second look surgery. Total abdominal irradiation has been given to patients with <5 mm residual disease. Fully 1/3 of all the patients entered on study are currently NED after chemotherapy $\pm$ irradiation. For patients with Stage III disease there is an

80% complete remission rate and the median survival has not been reached but will exceed 3 years. This short term aggressive regimen has substantial toxicity, particularly hematologic and peripheral neuropathy, and as soon as feasible we plan to utilize DDTC (see above) as a chemoprotector.

3) High-Dose CBDCA in Refractory Ovarian Cancer Patients: Because of the activity of high dose cisplatin in the treatment of refractory ovarian cancer, we initiated a trial of carboplatin (CBDCA), a cisplatin analogue, in patients who had failed primary induction therapy. CBDCA 400 mg/M^2 x 2 days (total of 800 mg/M^2) x 4 cycles was utilized. Over 50 patients have now entered clinical trial. The vast majority (92%) of patients had previously failed cisplatin therapy. In spite of previous therapy, objective responses were seen in 33% of patients. Dose limiting toxicity was thrombocytopenia and leukopenia. There was no peripheral neuropathy, ototoxicity and no renal toxicity. This platinum analogue is very active in ovarian cancer and has a different spectrum of dose-limiting toxicity than the parent drug.

Ovarian Carcinoma: Published:

1. Reed, E., Yuspa, S.H., Zwelling, L.A., Ozols, R.F., Poirier, M.C.: Quantitation of cisplatin-DNA-intrastrand adducts in testicular and ovarian cancer patients receiving cisplatin chemotherapy. J. Clin. Invest. 77: 545-550, 1986.

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Testicular Carcinoma: Established

1) Advanced Poor Prognosis Testicular Cancer - PVBV vs PVB: A four-drug combination, PVBV, composed of high dose cisplatin (40 mg/M^2 qd x 5), velban, bleomycin, and VP-16, was compared to conventional therapy in poor prognosis advanced non-seminomatous testicular cancer. Newly established hydration techniques prevented renal toxicity with high dose cisplatin. In a randomized comparison between the new therapy and standard treatment, there was a highly significant improvement in disease-free survival (68% vs. 33%) and a major benefit in overall actuarial 5 year survival (78% vs. 48%). Using this improved dose-intense chemotherapy regimen, it now appears that almost

80% of these patients with poor-prognosis advanced testicular cancer can now be cured. Testicular cancer is increasing in frequency according to SEER data (28% increase between 1975 and 1984) and there are now about 5000 new cases yearly. These results indicate that PVBV treatment of poor prognosis non-seminomatous testicular patients results in more patients alive and continuously disease-free than previously published standard regimens. The treatment of all poor prognosis testicular cancer patients with this new regimen would be expected to save 500 lives per year in the United States alone.

Testicular Carcinoma: Published

1. Reed, E., Yuspa, S.H., Zwelling, L.A., Ozols, R.F., Poirier, M.C.: Quantitation of cisplatin-DNA-intrastrand adducts in testicular and ovarian cancer patients receiving cisplatin chemotherapy. J. Clin. Invest. 77: 545-550, 1986.

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6. Ozols, R.F.: High dose chemotherapy in the treatment of testicular and ovarian cancer. In Proceedings of Beijing International Symposium on Cancer Therapy, 1987 (in press).

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AIDS and Kaposi's Sarcoma: Established

1) AIDS and Kaposi's Sarcoma: The Medicine Branch has continued its intramural clinical research effort in AIDS-Kaposi's sarcoma in support of the clinical trials underway under the direction of the Associate Director,

Clinical Oncology Program. The clinical support necessary to complete the important clinical trials on AZT and dideoxycytidine was provided by the Medical Staff Fellows in the Medicine Branch. In addition, 78 patients with AIDS-Kaposi's sarcoma have been entered on a series of clinical trials. First, we have documented the relatively modest activity of interferon in inducing Kaposi's sarcoma regressions and have documented the high frequency of infectious complications even in the interferon-treated patients. Subsequent trials randomizing patients to interferon on an alternating non-crossresistant combination chemotherapy regimen have demonstrated similar overall response rates and relatively short durations of remission. Infectious complications were similar in both arms of the trial. In addition, pilot trials of suramin, interferon, and IL-2 combination therapy for early stage AIDS and a pilot trial of recombinant alpha-interferon and DFMO in Kaposi's sarcoma and AIDS have been completed. Laboratory studies involve studies on corticosteroid-induced genes and HIV infection; gene activation or suppression by HIV transactivators; DNA repair alterations induced by AZT. Details of these studies can be found in the attached laboratory sections.

AIDS and Kaposi's Sarcoma: Published

1. Papadopoulos, N.M., Lane, H.C., Costello, R., Moutsopoulos, H.M., Masur, H., Gelmann, E.P., and Fauci, A.S.: Oligoclonal immunoglobulins in patients with the Acquired Immunodeficiency Syndrome. Clin. Immunol. Immunopath. in press.

2. Masur, H., Lane, H.C., Palestine, A., Smith. P.D., Manischewitz, J., Stevens, G., Fujikawa, L., Macher, A., Nussenblatt, R., Baird, B., Megill, M., Wittek, A., Quinnan, G., Parrillo, J.E., Rook, A., Eron, L., Poretz, D., Goldberg, R., Fauci, A., and Gelmann, E.P.: Effects of 9-(1,3 dihydroxy-2-propoxymethyl) guanine on serious cytomyelovirus disease in eight immunosuppresed homosexual men. Ann. Int. Med. 104: 41-44, 1986.

3. Stein, C., Saville, W., Yarchoan, R., Broder, S., and Gelmann, E.P.: Suramin and function of the adrenal cortex. Ann. Int. Med. 104: 286-287, 1986.

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Pheochromocytoma: Established

Historically no effective therapy has been available for malignant unresectable pheochromocytoma and patients often had uncontrollable hypertension, severe flushing as well as progressive malignant disease. We have developed a combination chemotherapy approach to this disease which has resulted in a 47% objective response rate in the first 15 patients. Not only has this three drug

regimen (cyclophosphamide, vincristine, and dacarbazine) produced objective regressions, but it has resulted in substantial control of hypertension and amelioration of symptoms in an additional 26% of patients. This combination chemotherapy approach represents the first effective therapy for metastatic pheochromocytoma ever described.

Pheochromocytoma: Published

1. Goldstein, D.S., Stull, R., Eisenhofer, G., Sisson, J.C., Weder, A., Averbuch, S., and Keiser, H.R.: Plasma levels of DOPA and catecholamines in patients with neuroblastoma or pheochromocytoma. Ann. Intern. Med. (in press).

2. Palestine, A., Stevens, G., Lane, H.C., Masur, H., Fujikawa, L., Nussenblatt, R.B., Rook, A., Manischewitz, J., Baird, B., Megill, M., Quinnan, G., Gelmann, E., and Fauci, A.S.: Treatment of cytomegalovirus retinitis with dihydroxy propoxymethyl guanine. Am. J. Opthalomol. 101: 95-101, 1986.

3. Averbuch, S.D., Steakley, C.S., Goldstein, D.S., Keiser, H.R. and Gelmann, E.P.: Treatment of malignant pheochromocytoma with cyclophosphamide, vincristine, and dacarbazine. Proc. ASCO 5: 135, 1986.

4. Goldstein, D.S., Stull, R., Eisenhofer, G., Sisson, J.C., Weder, A., Averbuch, S.D., and Keiser, H.R.: Plasma dihydroxyphenylalanine in neuroblastoma and pheochromocytoma. Clin. Res. 34: 479A, 1986.

5. Averbuch, S.D., Keiser, H.R., Wade, J.L., Goldstein, D.S. and Douglas, F.L.: Treatment of malignant pheochromocytoma with combination chemotherapy. Clin. Res. 32: 698A, 1984.

Breast Carcinoma:

Details of the clinical programs on breast cancer may be found with section entitled "Clinical Program in Breast Cancer".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO 1 CM 06119-18 M

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Cytogenetic studies

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

PI:	Jacqueline Whang-Peng	Senior Investigator	MB	NCI
Others:	Turid Knutsen	Med. Tech	MB	NCI
	Elaine Lee	Med. Tech	MB	NCI
	Chien-Song Kao-Shan	Staff Fellow	MB	NCI
	Yi-Fa Liu	Visiting Fellow	EEB	NCI
	Neil Caporaso	Medical Staff Fellow	EEB	NCI
	Karl Theil	Guest Worker		

COOPERATING UNITS *(if any)*

Cooperating Units: Environmental Epidimiology Branch, National Cancer Institute (NCI); Medical Oncology Branch, NCI; Clinical Pharmacology Branch, NCI; Pediatric Oncology Branch, NCI; Division of Cancer Etiology, NCI; (Cont.)

LAB/BRANCH

Medicine Branch

SECTION

Cytogenetic Oncology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	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.)*

AREAS OF INVESTIGATION

1. Cytogenetic studies of human neoplastic, hematologic, and congenital diseases, with special emphasis on patients with AIDS who develop leukemia, lymphoma, or Kaposi's sarcoma. Specific diseases studied inlude lymphoma (Burkitt's and non-Burkitt's), non-small cell carcinoma of the lung, rhabdomyosarcoma, renal cell carcinoma, and esophageal cancer.

2. Localization of MDR (multiple drug resistance) gene in cell lines from breast cancer (MCF-7), ovarian cancer, and in drug resistant (e.g. ADR, Colchicine) epidermoid cell lines (A431).

3. Localization of the following genes in normal chromosomes, using the in situ hybridization technique and normal human lymphocytes:
   Dr. K. Cowan - pA1, pADR1, melara, 19-1-2 (DNA & RNA probe)
   Dr. Matt Hentz - pst Ferritin, kpnI ferritin.
   Dr. I. Magrath - c-myc, v-sis.
   Drs. L. Liu and J. Wong - Z-II, C-I, pGTHI, GST
   Dr. E. Chang - Ha-ras 2, Ki-ras-1.

4. Fragile sites studies:
   a. In vitro studies of fragile sites in the bone marrow and peripheral blood of heavy cigarette smokers.
   b. Continuation of in vitro studies of fragile sites in peripheral blood chromosomes from individuals of different ages.
   c. Studies of fragile sites in the peripheral blood of high risk cancer families, e.g., melanoma, and dysplastic nevi syndrome.

John Minna	Branch Chief	MOB-NNMC	NCI
Kenneth Cowan	Sr. Staff	CPB	NCI
Mark Israel	Sr. Staff	POB	NCI
Ian Magrath	Sr. Staff	POB	NCI
Susan Sieber	Deputy Director	DCCP	NCI
Robert Fine	Clinical Associate	CPB	NCI
Yves Pommier	Visiting Associate	LMP,DCT	NCI
Timothy Triche	Sr. Staff	LP	NCI
Marston Linehan	Sr. Staff	C DCT	NCI

James Wong (Director- Dept. of Biochemistry & Molecular Biology Harvard Medical School)

Leroy Liu (Senior Staff, Dept. of Physiological Chemistry, Johns Hopkin Medical School)

Laboratory of Molecualr Pharmacology, NCI; Laboratory of Pathology, NCI; Surgery Branch, NCI; Harvard Medical School, Boston, Johns Hopkins Medical School, Baltimore.

5. DNA topoisomerase studies:
   a. Kinetics of Topoisomerase I and II in the cell cycle.
   b. Use of immunofluorescent labeled antibody to top II to detect the site of action in the chromosome.
   c. The relationship between topoisomerase II and fragile sites.
   d. Mapping of the human DNA topoisomerase I and II genes on normal lymphocyte chromosomes, using the in-situ hybridization technique.
   e. The effect of antitumor drugs (VP 16, Camptothecin, m-AMSA etc.) on DNA in relation to topoisomerase I and II.

PROJECTS COMPLETED:

1. Cytogenetic studies of human neoplastic, hematological diseases:
   a. 68 patients who developed secondary leukemia (SL)/dysmyelopoietic syndrome (DMS) following extensive chemotherapy and or radiation therapy as well as of patients who developed SL/DMS without such treatment showed that those patients who received radiation alone or with chemotherapy had more extensive numerical and structural abnormalities than those who received only chemotherapy. In terms of the specific chromosomal abnormalities, there were no differences between the various treatment groups. Hypodiploidy, loss of chromosome 7, and structural abnormalities involving chromosomes 3 and 5 were the most common findings. Serial studies revealed that cyt - ogenetic abnormaities do not precede the development of hematological changes by significant time periods. Paper submitted to Blood.
   b. Proposed origin of small cell lung cancer (SCLC): Abnormalities of chromosome 3p have been reported in patients with SCLC. Cytogenetic studies of two cases of secondary erythroleukemia occurring after treatment of SCLC showed the presence of the typical SCLC marker, del(3)(p14P23) in their leukemia cells originating from a stem cell capable of differentiating as well as macrophages and hematopoieti c cells.
   c. 14 Human esophageal carcinmoma cell lines were studied. Both numerical and structural chromosomal abnormalities were extensive in

these lines. The smallest overlapping region was noted on chromosome 11 at the band p13 in 12 of the lines.

2. Cytogenetic analysis of the Adr^R cell line (adriamycin resistant MCF-7 breast cancer cell line) showed the presence of homogeneously staining regions (HSRs) on chromosome 11 and rearranged chromosome 7. DNA sequences which were amplified 50-100 fold in the Adr^R cell line and which covered over a total of 140 kb were isolated. In situ hybridization studies demonstrated that this sequence was found on chromosome 7q21.1 in normal human lymphocytes and that some amplified DNA sequences isolated by the in-gel hybridization technique were linked to this sequence in the HSRs in the Adr^R cells.

3. Fragile sites:
We examined the peripheral blood and bone marrow in 18 smokers, (15 females and 3 males) with a median age of 25 (21-40), and an average cigarette use corresponding to 6 pack years. In both bone marrow cells and peripheral blood lymphocytes, we were able to show a significantly increased frequency of SCEs in smokers whose smoking history equalled 5 or more cigarette pack years, but not in those who smoked less. We also found a higher frequency of SCEs in peripheral blood lymphocytes than in bone marrow cells. We also showed a significantly higher frequency of fragile sites in peripheral blood lymphocytes of smokers and elevated expressions of fragile sites at the cancer breakpoints, 3p14.2, 11q13.3, 22q12.2 and 11p13-14.2. Studies of the chromosomal changes in these cells may make it possible to assess the mutagenic damage caused by these exogenous agents in individual patients, and may predict susceptibility to malignant events.

4. DNA topoisomerase I and II:
   a. Kinetic studies:
      Topo I: constantly appears throughout the cell cycle, in both stimulated and non-stimulated lymphocytes, and in Hela cells.
      Topo II: top II is not present in resting cells (phase G_0 - G^1). It first appears at the middle part of S phase and highest concentrations are present in S to G_2, and M.
   b. Use of top II antibody to detect the site of action:
      Lymphocytes and HeLa cell chromosome stained evenly with immunofluoresent stains, revealing no distinct banding patterns.
   c. Relationship between top II and fragile sites:
      Human lymphocyte chromosomes were labeled with ^3H-thymidine and then subjected to the fragile site harvesting procedure. The cleavable complexes were trapped with SDS and the protein:DNA complexes analyzed to determine if top II is the protein involved in these complexes. Unfortunately, the amount of trapped protein was too small to detect the presence of topII in the DNA complex. It has been proposed that the fragile site is a nucleosome free region. In collaboration with Dr. Berton Zbar (FCRF, NCI) we plan to study the D3S2 probe, a polymorphic allele to MspI.

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2. Trent, J.M., Crickard, K., Gibas, Z., Goodacre, A., Pathak, S., Sandberg, A., Thompson, F., Whang-Peng, J., Wolman, S.: Methodologic advances in the cytogenetic analyses of human solid tumors. Cancer Genet. Cytogenet. 19:57-66, 1986.

3. Behrens, B.C., Hamilton, T.C., Grotzinger, K.R., Whang-Peng, J., Batist, G., Louie, K.G., Knutsen, T., McKoy, W.M., Young, R.C., Ozols, R.F.: Characterization of a cisplatin-resistant human ovarian cancer cell line and its use in evaluation of cisplatin analogs. Cancer Res. 47:414-418, 1987.

4. Thiele, C.J., Whang-Peng, J., Kao-Shan, C-S., Gelmann, E.P., Miser, J., Israel, M.A.: Translocation of c-sis proto-oncogene in peripheral neuro-epithelioma. Cancer Genet. Cytogenet. 24:119-128, 1987.

5. Whang-Peng, J., Knutsen, T.: Cytogenetic studies in neoplasms (human and animal): implications, prognosis, and treatment. In: Progressive Stages of Malignant Neoplastic Growth, H.E. Kaiser (Ed.), Martinus Nijhoff, Dordrecht, In Press.

6. Whang-Peng, J., Lee, E.C., Kao-Shan, C-S., Schechter, G.: Ring chromosome in a case of acute myelomonocytic leukemia: its significance and a review of the literature. Hematol. Pathol. 1:57-65, 1987.

7. Whang-Peng, J., Knutsen, T.: The role of cytogenetics in the characteriz ation of acute leukemia: Acute lymphoblastic leukemia and acute myelo blastic leukemia. In: Acute Leukemia: Characterization and Diagnosis, S.A. Stass (Ed.), Marcel Dekker, Inc., New York (In Press).

8. Bloomfield, C.D., Goldman, A.I., Alimena, G., Berger, R., Borgstrom, G.H., Brandt, L, Catovsky, D., de la Chapelle, A., Dewald, G.W., Garson, O.M., Garwicz, S., Golomb, H.M., Hossfeld, D.K., Lawler, S.D., Mitelman, F., Nilsson, P., Pierre, R.V., Phillip, P., Prigogina, E., Rowley, J.D., Sakurai, M., Sandberg, A.A., Secker-Walker L.M., Tricot, G., Van Den Berghe, H., Van Orshoven, A., Vuopio, P., and Whang-Peng, J: Chromosomal abnormalities identify high-risk and low-risk patients with acute lympho-blastic leukemia. Blood 67:415-420, 1986.

9. Kao-Shan, S.C., Fine, R.L., Whang-Peng, J., Lee, E., Chabner, B.S.: Fragile sites and increased sister chromatid exchanges (SCEs) in bone marrow and peripheral blood of young cigarette smokers. In: Proceed First Ann. Meet. Chin. Am. Profess. Asso., Washington, D.C., 1986, pp. 55-66.

10. Jones, D.B., Kaye, F., Hardman, J., Whang-Peng, J., Dale, N.L., Garvin, D.F., Schumacher, H.R.: The eosinophilic variant of acute myelomonocytic leukemia developing as a secondary leukemia in a patient with mycosis fungoides. Hematol. Pathol. 1:67-76, 1987.

11. Orman, S.V., Schechter, G.P., Whang-Peng, J., Guccion, J., Chan, C., Schulof, R.S., Shalhoub, R.J.: Nephrotic syndrome associated with a clonal T-cell leukemia of large granular lymphocytes with cytotoxic function. Arch. Intern. Med. 146:1827-1829, 1986.

12. Abbondanzo, S.L., Gray, R.G., Whang-Peng, J., Jacobson, R.J.: A myelo dysplastic syndrome with marrow eospinophilia terminating in acute nonlymphocytic leukemia, associated with an abnormal chromosome 16. Arch. Pathol. Lab. Med. 111:330-332, 1987.

13. Whang-Peng, J., Longo, D., Lee, E.C., Gazdar, A.D., Young, R.C.: Proposed origin of small cell lung cancer. (In preparation).

14. Whang-Peng, J., Young, R.C., Lee, E.C., Longo, D.L., Schechter G.P., Devita, V.T.: Cytogenetic studies in patients with secondary leukemia/ dysmyelopoietic syndrome following different treatment modalities. (Submitted to Blood).

15. Whang-Peng, J., Schlegel, S.P., Lee, E.C.: Cytogenetic studies in esophageal carcinoma cell lines. (In preparation).

16. Whang-Peng, J., Lee, E.C.: Cytogenetics. In: The Non-Hodgkin's Lymphomas, I. Magrath (Ed.), Edward Arnold, Ltd., London. (In preparation).

17. Fairchild, C.R., Ivy, S.P., Kao-Shan, C.S., Whang-Peng, J., Rosen, N., Israel, M., Melera, P.W., Cowan, K.H.,, Goldsmith, M.E.: Isolation of amplified and over-exposed DNA sequences from adriamycin-resistant human breast cancer cells. Cancer Research. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06700-14 M

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Clinical Program in Breast Cancer

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

PI:	Marc E. Lippman	Senior Investigator	M	NCI
Other:	Sandra Swain	Senior Staff Fellow	M	NCI
	Elizabeth Egan	Nurse	M	NCI
	Judith Bader	Radiotherapist	ROB	NCI
	Helene Smith	Collaborator	Peralta	CA

COOPERATING UNITS *(if any)*
Radiation Oncology Branch, NCI; Surgery Branch, NCI;
Peralta Cancer Research Institute, CA.

LAB/BRANCH: Medicine Branch and Division of Cancer Control and Rehabilitation

SECTION: Medical Breast Cancer Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3 1/2	2 1/2	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 Medical Breast Cancer Section is responsible for the development of a clinical and laboratory program directed at breast cancer. Clinical trials in metastatic disease comparing chemotherapeutic, hormonal and chemohormonal regimens are underway. Biochemical and hormonal receptor studies are undertaken and coordinated by the Medical Breast Cancer Section. Clinical studies consist of a major chemotherapy trial aimed at stimulating human breast cancer cells with hormonal agents for more successful cell cycle phase specific chemotherapy; a hormonal therapy trial aimed at prospectively evaluating the usefulness of complete endocrine ablation in human breast cancer. Concurrent cytokinetic data are being collected. Phase II trials of CBDCA and leukovorin followed by 5-Fu are underway. We have developed a successful treatment program for Stage III breast cancer (objective response rate 94%) (51% CR rate). We are attempting to further refine these techniques. We have initiated a randomized trial to explore the usefulness of an in vitro chemosensitivity assay system in collaboration with Helene Smith, Ph.D. (Peralta Cancer Research Institute). A trial for Stage IV no evidence of disease patients continues. In addition there is an endocrine and chemotherapy program for male breast cancer. A cooperative trial between the Surgery, Radiation and Medicine Branches is underway comparing excisional biopsy plus definitive radiotherapy to simple mastectomy in clinical Stage I and II breast cancer. All patients have axillary dissections; A-C chemotherapy is given to all N+ patients; 232 patients are on study.

Project Description:

The Medical Breast Cancer Service was established in July 1972, and the clinical program was initiated in January 1973. It was responsible to the Office of the Associate Director, COP, until its shift to the Medicine Branch in August 1974.

I. Clinical Trials

A. Recurrent disease trials.

1. We have initiated a trial using "synchronization" techniques for patients with Stage III locally advanced breast cancer. 90 patients are on study. Objective response rate is 94%; 54% of patients have complete responses to chemotherapy alone. This study has been extended to patients with Stage IV metastatic disease.

2. We have begun a randomized trial using a novel in vitro culture system to assess drug sensitivity. We have achieved nearly a 2/3 success rate in obtaining drug sensitivity information using this assay.

3. A primary endocrine trial studying total endocrine ablation with leuprolide, tamoxifen and aminogluthethimide has been initiated.

4. A protocol for sequential endocrine approaches to male breast cancer with concurrent receptor analyses are ongoing. A trial of adjuvant therapy of Stage II MBC is also underway. 26 patients are on study. 5 patients have relapsed. Actuarial 5 year disease free survival is 80%.

5. A randomized trial of radical radiation therapy versus simple mastectomy is underway with 232 patients on study. Thus far, DF survival and overall survival are identical.

II. Extramural Activities

A. National Surgical Adjuvant Breast Project

Dr. Lippman is on the Endocrine and Stage III Committee of the National Surgical Adjuvant Breast Project.

B. Outside Teaching Responsibilities

Dr. Lippman is Clinical Professor of Medicine and Pharmacology at the USUHS Medical School.

Selected Publications:

1. Lippman, M.E., Sorace, R.A., Bagley, C.S., Danforth, D.W., Findlay, P. and Wesley, M. Treatment of locally advanced breast cancer using primary induction chemotherapy with hormonal synchronization followed by radiation therapy with or without debulking surgery. NCI Monographs, 1: 153-159, 1986.

2. Lippman, M.E., Edwards, B.K., Findlay, P., Danforth, D., MacDonald, H., d'Angelo, T., Gorrell, C. The influence of definitive radiation therapy for primary breast cancer on ability to deliver adjuvant chemotherapy. NCI Monographs 1: 99-104, 1986.

3. Lippman, M.E.: National Institutes of Health Consensus Development Conference on Adjuvant Chemotherapy for Breast Cancer - A Commentary. Breast Cancer Res. Treat. 6(3): 195-200, 1985.

4. Dickson, R.B., Kasid, A., Huff, K., Bates, S.E., Knabbe C., Bronzert, D., Gelmann, E. and Lippman, M.E. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17B-estradiol or v-Ha-ras oncogene. Proc. Natl. Acad. Sci. 84: 837-841, 1987.

5. Swain, S., Sorace, R.A., Bagley, C.S., Danforth, D.W., Bader, J., Wesley, M.N., Steinberg, S. and Lippman, M.E. Neoadjuvant chemotherapy in the combined modality approach of locally advanced nonmetastatic breast cancer. Cancer Res. (in press) 1987.

6. Chiedozi, L.C., Huff, K.K., Wesley, M.N. and Lippman, M.E. Estrogen receptors in advanced primary breast cancer. Nigerian Cancer Society, East African Med. J. 62: 26-31, 1985.

7. Lippman, M.E. Steroids in malignant diseases: progress in patient selection. Hospital Practice 93-106, 1984.

8. Bagley, C.S., Wesley, M.N., Young, R.C. and Lippman, M.E. Adjuvant therapy of male breast cancer. Am. J. Clin. Oncol. 10(1): 55-60, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06702-12 M

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Mechanisms of Hormone Dependence of Human Malignancy

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

PI:	Marc E. Lippman	Senior Investigator	M	NCI
Other:	Edward Gelmann	Senior Investigator	M	NCI
	Neal Rosen	Senior Investigator	M	NCI
	Diane Bronzert	Technician	M	NCI
	Karen Huff	Technician	M	NCI
	Susan Aitken	Technician	M	NCI
	Robert Dickson	Senior Investigator	M	NCI

(continued on next page)

COOPERATING UNITS *(if any)*: Laboratory of Biochemistry, NCI

LAB/BRANCH: Medicine Branch

SECTION: Medical Breast Cancer Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	12	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A. We are studying the molecular mechanisms by which estrogens and antiestrogens specifically alter growth of human breast cancer.

1. We have introduced viral and cellular onc genes (ras and myc) into human breast cancer cells. These retroviruses are stably integrated and viral mRNA is expressed at high levels. Ras induces a hormone independent phenotype. This occurs through increased secretion of several specific growth factors in an autonomous fashion.

2. We are using the technique of differential hybridization to identify specific estrogen regulated genes for cloning and subsequent analysis. We have identified several unique genes induced within 6 hours by estradiol. We have also identified a gene which is specifically induced by antiestrogens and de-induced by estrogen stimulation.

3. We have identified and partially purified several estrogen induced growth factors which are secreted by breast cancer cells into the medium. These include TGFα, EGF, IGF-1, TGFβ, PDGF a and PDGF b. In addition we have identified a new $\sim$60K growth factor which stimulates clonogenic proliferation of epithelial cells. A very extensive series of investigations are underway probing the range of expression, regulation and pathophysiologic significance of these activities. Among the techniques we are using are antibodies against these growth factors or their receptors, antisense transfections, and growth factor antagonists.

Lance Liotta	Chief	DCBD	LP
George Martin	Chief	LDBA	NIDR
William Kidwell	Chief	CCRS	DCBD
David Salomon	Research Biochemist	DCBD	LTIB
Snorri Thorgeirsson	Chief	LEC	DCE
George Wilding	Senior Staff Fellow	M	NCI
Susan Bates	Guest Worker	M	NCI
Gerhart Zugmaier	Guest Worker	M	NCI
Eva Valverius	Visiting Fellow	M	NCI
Miguel Saceda Sanchez	Guest Worker	M	NCI
Mary Beth Martin	Biotechnology Fellow	M	NCI
Robert Clarke	BCSG Fellow	M	NCI
Bruce Ennis	BCSG Fellow	M	NCI

Publications

1. Knabbe, C., Lippman, M.E., Wakefield, L.M., Derynck, R. and Dickson, R.B. Evidence that transforming growth factor is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428, 1987.

2. Albini, A., Graf, J., Kitten, T., Kleinman, H.K., Martin, G.R., Veillette, A. and Lippman, M.E. 17B-estradiol and ^{H}v-ras transfection increses invasiveness of MCF-7 breast cancer cells. PNAS 83: 8182-8186, 1986.

3. Dickson, R.B., Bates, S., McManaway, M. and Lippman, M.E. Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res. 46: 1707-1713, 1986.

4. Dickson, R.B. and Lippman, M.E. Estrogen regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocrine Reviews 8(1): 29-43, 1987.

5. Lippman, M.E., Dickosn, R.B., Bates, S., Knabbe, C., Huff, K., Swain, S., McManaway, M., Bronzert, D., Kasid, A., and Gelmann, E.P. Autocrine and paracrine growth regulation of human breast cancer. Breast Cancer Res. Treat. 7: 59-70, 1986.

6. Davidson, N.E., Bronzert, D.A., Chambon, P., Gelmann, E. and Lippman, M.E. Use of two MCF-7 cell variants to evaluate the growth regulatory potential of estrogen-induced products. Cancer Res. 46: 1904-1908, 1986.

7. Lippman, M.E., Dickosn, R., Kasid, A., Gelmann, E., Davidson, N., McManaway, M., Huff, K., Bronzert, D., Bates, S., Swain, S., and Knabbe, C. Autocrine and paracrine growth regulation of human breast cancer. J. Steroid Biochem. 24(1): 147-154, 1986.

8. Huff, K.K., Kaufman, D., Gabbay, K.H., Spencer, E.M., Lippman, M.E. and Dickson, R.B. Secretion of an insulin-like growth factor I-related protein by human breast cancer cells. Cancer Res. 46(9): 4613-4619, 1986.

9. Lippman, M.E., Huff, K.K., Jakesz, R., Hecht, T., Kasid, A., Bates, S., and Dickson, R. Estrogens regulate production of specific growth factors in hormone dependent human breast cancer. Endocrinology of the Breast: Basic and Clinical Aspects. Annals N.Y. Acad. Sci. A. Angeli, H.L. Bradlow and L. Dogliotti (eds) pp. 11-16, 1986.

10. Bronzert, D.A., Davidson, N. and Lippman, M.E. Estrogen and antiestrogen resistance in human breast cancer cell lines. Adv. Exp. Med. Biol. 196: 329-345, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06709-07 M

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Mechanisms of Drug Resistance

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

PI:	Robert F. Ozols	Sr. Investigator	M	NCI
Other:	Robert C. Young	Chief	M	NCI
	Antonio Fojo	Sr. Investigator	M	NCI
	Karen Grotzinger	Med Technologist	M	NCI
	Thomas C. Hamilton	Sr. Staff Fellow	M	NCI
	Mary Ann Pritting	Chemist	M	NCI
	G-Ming Lai	Guest Researcher	M	NCI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Biology, DCBD
Laboratory of Cellular Carcinogenesis and Tumor Promotion, DCT

LAB/BRANCH

Medicine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	5	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.)*

We are studying the biology of ovarian cancer, the mechanisms of antineoplastic drug resistance in ovarian cancer, and the pharmacologic reversal of the drug resistant phenotype. We have characterized 7 new ovarian cancer cell lines including a line which has steroid hormone receptors. Drug resistant variant cell lines have been developed which are 6-100 more resistant to chemotherapy than the sensitive parental cell lines. An intraperitoneal model of human ovarian cancer was developed with ascites, pulmonary metastases, and death from intraabdominal carcinomatosis. Using these model systems we have demonstrated that resistance to melphalan, cisplatin, and adriamycin is linked, in part, to glutathione levels. We have shown that buthionine sulfoximine (BSO), a synthetic amino acid which inhibits the synthesis of glutathione, leads to a decrease in glutathione levels in the drug resistant cell lines and increases the cytotoxicity of melphalan, cisplatin and adriamycin. BSO plus melphalan also prolongs survival in the nude mouse system of ovarian cancer. In addition, we have demonstrated that some drug resistant human ovarian cancer cell lines have a decreased accumulation of adriamycin which can be reversed by exposure of the cells to verapamil. These studies led to a trial of verapamil plus adriamycin in refractory ovarian cancer patients and the results with BSO have led to the preclinical evaluation of BSO by the Decision Network of the NCI. We have also shown that DNA-repair is an important mechanism of resistance to cisplatin and melphalan in human ovarian cancer cells. Aphidicolin, an inhibitor of DNA polymerase increases the cytotoxicity of cisplatin in the resistant cell lines. The molecular basis for cisplatin resistance is being investigated with the goal of identifying and cloning the responsible gene.

GPO 914-918

1. Model Systems of Human Ovarian Cancer. We have established 7 long-term cell lines of human ovarian cancer. Drug resistant cell lines have been established from drug sensitive cell lines by exposure to antineoplastic agents in vitro. In vitro and in vivo selection procedures were used to establish a transplantable human ovarian cancer in nude mice which produces ascites and death from intraabdominal carcinomatosis. Drug resistant variants are being established in vivo in this model system.

2. Mechanisms Of Drug Resistance. We are examining the mechanisms of drug resistance to melphalan, adriamycin, and cisplatin in experimental models of human ovarian cancer. Studies are in progress to evaluate the role of GSH in the formation of DNA-cisplatin adducts, and in the repair of DNA damage in drug resistant cell lines. We have demonstrated that cisplatin and melphalan resistance is associated with increased DNA repair. Studies are in progress to determine the role of gene amplification and expression in the drug resistant cell lines.

3. Alterations Of Drug Resistance. We are examining the role of potential modifiers of drug resistance in human ovarian cancer cell lines. Manilations of glutathione alter sensitivity to melphalan, cisplatin, and adriamycin. We have demonstrated that buthionine sulfoximine plus melphalan prolongs survival in the nude mouse model compared to treatment with melphalan alone providing further evidence that GSH modulations may be clinically feasible. Furthermore, inhibition of DNA polymerase with apidicolin decreases DNA repair capacity and potentiates the cytotoxicity of cisplatin in drug resistant cell lines.

5. Immunotherapy of Ovarian Cancer. In collaboration with Dr. Ira Pastan, Laboratory of Molecular Biology, DCBD we have demonstrated that monoclonal antibodies linked to toxins (pseudomonas, ricin) are cytotoxic to human ovarian cancer cell lines. Clinical protocols have been initiated based on these observations.

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4. Reed, E., Yuspa, S.H., Zwelling, L.A., Ozols, R.F., Poirier, M.C.: Quantitation of cisplatin-DNA-intrastrand adducts in testicular and ovarian cancer patients receiving cisplatin chemotherapy. J. Clin. Invest. 77: 545-550, 1986.

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6. Ortaldo, J.R., Porter, H.R., Miller, P., Stevenson, H.C., Ozols, R.F., and Hamilton, T.C.: Adoptive cellular immunotherapy of human ovarian carcinoma xenografts in nude mice. Cancer Res. 46: 4414-4419, 1986.

7. Louie, K.G., Hamilton, T.C., Behrens, B.C., Grotzinger, K.R., McKoy, W.M., Myers, C.E., Young, R.C., and Ozols, R.F.: Intraperitoneal Aclacinomycin A for the treatment of human ovarian carcinoma. Cancer Chemother. & Pharm. 18: 153-156, 1986.

8. Behrens, B.C., Hamilton, T.C., Grotzinger, K.R., Whang-Peng, J., Batist, G., Louie, K.G., Knutsen, T., McKoy, W.M., Young, R.C., and Ozols, R.F.: Characterization of a cisplatin-resistant human ovarian cancer cell line and its use in evaluation of cisplatin analogs. Cancer Res. 47: 414-418, 1987.

9. Fitzgerald, D.J., Bjorn, M.J., Ferris, R.J., Winkelhake, J.L., Frankel, A.E., Hamilton, T.C., Ozols, R.F., Willingham, M.C., and Pastan, I.: Anti-tumor activity of an immunotoxin directed against the transferrin receptor of human ovarian cancer cells in nude mice. Cancer Res. 47: 1407-1410, 1987.

10. Ozols, R.F., Cunnion, R.E., Klecker, R.W. Jr., Hamilton, T.C., Ostchega, Y., Parrillo, J.E., and Young, R.C.: Verapamil and adriamycin in the treatment of drug resistant ovarian cancer patients. J. Clin. Oncol. 5: 641-647, 1987.

11. Reed, E., Ozols, R.F., Jarone, R., Yuspa, S.H., and Poirier, M.C.: Platinum-DNA adducts in nucleated peripheral blood cell DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. Proc. Natl. Acad. Sci. U.S.A., in press, 1987.

12. Ozols, R.F., Louie, K.G., Plowman, J., Behrens, B.C., Fine, R.L., Dykes, D., and Hamilton, T.C.: Enhanced alkylating agent cytotoxicity in human ovarian cancer in vitro and in tumor bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochem. Pharmacol. 36: 147-153, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06710-05 M

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Biology of Hormone-Responsive Human Malignancies

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

Edward Gelmann	Senior Investigator	M	NCI
Connie Agnor	Microbiologist	M	NCI
Carl Freter	Medical Staff Fellow	M	NCI
Susan Heckford	Visiting Associate	M	NCI
Francis Kern	Senior Staff Fellow	M	NCI

COOPERATING UNITS (if any)

Department of Pharmacology, USUHS

LAB/BRANCH: Medicine Branch

SECTION: Medical Breast Cancer Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	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.)

A. Our laboratory has been studying the molecular biological aspects of signals that trigger the malignant phenotype of human breast cancer cells. We have used the MCF-7 human breast cancer cell line as a model for hormone-dependent mammary tumorigenesis. These cells require exogenous estradiol supplementation for maximal in vitro growth rate and in vivo tumorigenecity. Using DNA transfection, we have introduced the v-ras^H oncogene into the MCF-7 cells and have shown that the expression of this activated gene results in the bypass of these cells' hormone dependence. We have followed up these studies by creating derivative cell lines with different c-ras^H mutants which differ in their malignant potential. Detailed studies of the derivative cell lines have shed light on the interaction between the c-ras^H gene and tumorigenesis of breast cancer cells. In our experiments, only a v-ras^H gene was capable of conferring tumorigenecity on human breast cancer cells. Other ras gene mutants and high levels of expression of c-ras^H did not alter the tumorigenetic properties of breast cancer cells. A second oncogene which may play a role in human breast cancer is the erbB oncogene which codes for a truncated EGF receptor protein. It appears that the expression of the c-erbB gene is inversely related to hormone dependence in many human breast cancer cell lines. We have constructed an MCF-7 derivative cell line transfected with the v-erbB gene to characterize a breast cancer cell with a constitutively activated EGF receptor and assess the effect of this on the hormone dependence and tumorigenesis potential of the cells.

GPO 914-914

George Wilding	Medical Staff Fellow	M	NCI
Maria Zajac-Kaye	Biotechnology Fellow	M	NCI
Nancy Davidson	Assistant Professor		

Department of Oncology Johns Hopkins University School of Medicine

B. We are isolating genes whose expression is characteristic of different stages of malignant progression of breast cancer. One such gene codes for the intermediate filament protein, vimentin, which is expressed in approximately half the cell lines representing hormone-independent breast cancer, but not in cell lines representing hormone-dependent breast cancer. This phenomenon of abnormal intermediate filament gene expression is accompanied by down-regulation of the native intermediate filament protein, in the case of mammary epithelial cells, cytokeratin. Experiments thus far suggest that this phenomenon is particular to the hormone-dependent malignancies, breast and prostate cancer, and cannot be demonstrated in other epithelial malignancies. We continue to isolate estrogendependent genes from hormone-dependent human breast cancer cell and are focusing on genes whose expression can confer phenotypic changes in our hormone-dependent breast cancer cell system.

C. Studies of a second human hormone-dependent malignancy, prostate cancer have been initiated utilizing human prostate cancer cell lines. We have characterized the androgen and antiandrogen responsiveness of a single hormonedependent cell line, and studied in detail the response of human prostate cancer cells to both androgen hormones and peptide growth factors.

PUBLICATIONS

1. Heckford, S.E., Gelmann, E.P., Agnor C.L..., Jackson, S., Zinn, S., Matis, L.A. Distinct signals are required for proliferation and lymphokine gene expression in murine T cells clones. J. Immunol., 137: 3652-3663, 1986.

2. Dickson, R., Kasid, A., Huff, K., Bates, S.E., Knabbe, C., Bronzert, D., Gelmann, E.P., Lippman, M.E. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 -estradiol in v-ras^{H} oncogene. Proc. Natl. Acad. Sci., USA, 84:837-841, 1987.

3. Davidson, N.E., Gelmann, E.P., Lippman, M.E., Dickson, R.B. Epidermal growth factor receptor gene expression in estrogen-receptor positive and negative human breast cancer cell lines. Molec. Endocrin. 1:216-223, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06712-02 M

PERIOD COVERED: September 1986 - May 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Genetic Control of Human Colon and Breast Cancer

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

PI:	Neal Rosen	Senior Investigator	M	NCI
Others:	Andre Veillette	Medical Staff Fellow	M	NCI
	Douglas Yee	Medical Staff Fellow	M	NCI
	Francine Foss	Medical Staff Fellow	M	NCI
	Frieda Bostick-Burton	Technician	M	NCI

COOPERATING UNITS (if any)
Laboratory of Tumor Virus Biology, NCI

LAB/BRANCH
Medicine Branch

SECTION
Medical Breast Cancer Section

INSTITUTE AND LOCATION
NCI/NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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.)

Over this period, our work has concentrated on the role of members of the c-src family of protooncogenes in the development of human malignancies and the identification and characterization of other gene products associated with the progression of human breast or colon carcinoma. This research can be subdivided into several projects.

1. Characterization of human protooncogenes in the c-src family. c-src, the protooncogene homolog of the Rous sarcoma virus oncogene v-src, is the prototypic member of a family of genes that encode membrane-bound, N-terminal myristylated, tyrosine protein kinases. The group, of which seven members have been identified, is characterized by highly conserved genes with common intron-exon structures, all encoding membrane bound, N-terminal myristylated tyrosine protein kinases of molecular weight 56-60 Kd.

Although three members of this family, src, yes, and fgr, were originally isolated as the transforming genes of RNA tumor viruses, there has been little evidence associating these gene products with human tumors. Our previous work has concentrated on defining the role of the c-src protooncogene in human tumors. We have shown that the c-src protein product ($pp60^{c\text{-}src}$) is specifically overexpressed in all normal and neoplastic tissues and cell lines of neuroectodermal origin that retain a neuronal phenotypoe. When immature neuroblastomas are differentiated in cell culture toward a neuronal pattern of cellular functions, there is an attendant increase in $pp60^{c\text{-}src}$ expression and total activity. As there is no obvious difference in expression between neuroblastoma and normal brain and no elevation of the specific activity of neural pp60c-src, this phenomenon is felt to be a marker of neural differentiation, rather than transformation of these cells.

Other types of normal cells and most human neoplasms have low levels of $pp60^{c-src}$ protein. There are several exceptions, however; we find that cell lines derived from breast and colon carcinoma have consistent elevations of $pp60^{c-src}$ protein kinase levels. In breast cancer, this elevation is accounted for by an increase in $pp60^{c-src}$ protein. Thus, total, but not specific activity is increased. Very low levels are detected in normal breast and fibrocystic breast tissue, fibroadenomas have somewhat elevated levels, and tumors have 20-50 fold higher levels of $pp60^{c-src}$ protein than normal tissue. Elevation of the expression of this gene is, therefore, closely associated with neoplastic growth of breast epithelia. Current studies are focused on investigating the functional consequences of this phenomenon.

We have also previously shown that colon carcinoma cell lines and tissue specimen have uniformly elevated levels of $pp60^{c-src}$ kinase activity compared to normal colonic mucosal controls. Twenty-one colon carcinoma cell lines have an average of 120 fold elevation over normal primary cultures. In these cell lines, c-src mRNA concentrations and $pp60^{c-src}$ protein content are increased 4-6 fold, so that the specific activity of the $pp60^{c-src}$ kinase is increased 20-fold. This tumor-specific increase is equivalent to that seen in animal cells transformed by the Rous sarcoma or polyoma viruses and suggests that c-src activation may play an important role in the development of colon carcinoma.

Our current research in this area includes investigations into the mechanism of c-src activation in tumors, the functional consequences of such activation, and the potential roles of other members of the c-src family in the development of human neoplasia. Progress in these areas over the past year and current lines of investigation are described below.

A. Activation of $pp60^{c-src}$ in human colon carcinoma. We have used *in vitro* translation of colonic c-src mRNA to show that $pp60^{c-src}$ protein kinase activity is inhibited by post-translational modification(s) that occur in normal colon cells. Presumably, the tumor cell enzyme is not susceptible to this inhibition, because of either a change in its structure or a change in other cellular regulatory enzymes.

Other workers have recently found that phosphorylation of c-src residue tyrosine 527 is associated with low enzyme activity and that activation of c-src by polyoma middle T antigen is accompanied by dephosphorylation of this residue. Furthermore, site-directed mutagenesis resulting in deletion of tyr 527 or substitution of phenylalanine for tyrosine at this position results in activation of $pp60^{c-src}$ kinase activity and causes the protein to become transforming.

We have found that in colon tumor cells $pp60^{c-src}$ tyr 527 is phosphorylated. However, the turnover of tyr 527 phosphate is 8-10 faster on $pp60^{c-src}$ from tumor cells than on that isolated from normal colonic mucosal cells. We postulate that this increased turnover is responsible for the elevated specific activity of $pp60^{c-src}$ derived from colon tumors. We have developed a model of activation which predicts that $pp60^{c-src}$ dephosphorylated on tyr 527 is in an active form and that rephosphorylation of this residue causes the molecule to slowly shift to an inactive conformation. Rapid turnover of tyr 527 phosphate would not allow this slow shift prior to dephosphorylation.

We have preliminary, indirect evidence for this hypothesis in that pH 6 will also activate $pp60^{c-src}$ kinase and reequilibration at pH 7 will result only in a slow diminution of activity.

Ongoing studies in the lab are focused on further defining the mechanism and consequences of $pp60^{c-src}$ activation in colon cancer. RNA mapping techniques and cloning and sequencing studies will be used to analyze the primary sequence of $pp60^{c-src}$ in colon cell lines. The enzymes responsible for specific tyr 527 phosphorylation are being sought; assays for $pp60^{c-src}$ tyr 527 phosphatase activity show no difference between normal and neoplastic colon cells.

In situ hybridization procedures are being developed to assess, with biochemical assays, at what point in the natural history of colon neoplasia $pp60^{c-src}$ becomes activated. Human wild type and tyr 527 deleted c-src will be transfected into normal colonic epithelial cells to assess the effects on growth of c-src activation in this system. In addition, putative antisense inhibitors of $pp60^{c-src}$ expression and peptide and natural product inhibitors of kinase activity are being tested in an attempt to establish systems in which the results of reducing $pp60^{c-src}$ activity can be assayed (see 1C, below)

B. Expression of other c-src related tyrosine kinase genes in human colon carcinoma. Hybridization of a v-src probe to a Northern blot of colon carcinoma mRNA reveals 2 bands of 4.0 and 3.0 kb and, in one cell line, a third band at 2.2 kb. The 4.0 kb band represents mRNA encoding human c-src. We have constructed a cDNA library from the cell line displaying all three bands and have isolated clones representing the 3.0 and 2.2 kb bands.

Sequencing the clones reveals the 3.0 kb band encodes a novel protein tyrosine kinase of 59 kd belonging to the c-src subfamily on the basis of intron-exon structure and overall homology. Other groups have also recently cloned this gene from normal human cells and it has been designated as c-fyn.

We have determined that this gene is expressed widely in a variety of normal cell types and like c-src is most highly expressed in normal neuronal cells and tissue. Normal colonic mucosal cells in culture express c-fyn at low levels compared to colon carcinoma cell lines. Twenty-one of these all overexpress the gene an average of 20-40 fold. Thus, like c-src, this gene is uniformly aberrently regulated in colon carcinoma cell lines, but unlike c-src, this is at the level of mRNA expression. We are in the process of raising specific antibodies in order to study the regulation of c-fyn associated enzyme activity.

The clones hybridizing to the 2.2 kb band were sequenced and were found to have a 98% amino acid homology to the murine lck gene and is the likely human counterpart. Murine lck is another member of the c-src family, encoding a 56 kd tyrosine kinase, and remarkable in rodents for stringent restriction of expression: lck mRNA is found only in normal and neoplastic murine T cells.

Using our human cDNA clones, we have found that the pattern of expression of lck in human cells is similar; normal T-cells, T-cell lymphomas, and some B-cell lymphomas express modest to large amounts of mRNA, whereas all other normal cell and tissue types and a wide variety of tumor cell types, including breast, ovarian, and non-small cell lung carcinomas, melanoma, neuroblastoma, and myelogenous leukemias express no lck.

The cDNA was cloned from a colon carcinoma, and when we screened colon carcinoma cell lines we found that 42% (9/22) expressed the gene. Of those cell lines derived from primary lesions, 2/14 were positive, those derived from metastases or ascites were all positive (7/7) some at levels exceeding those found in T-cell lymphomas. The only other tumor type in which we found a significant degree of lck expression is small cell lung carcinoma. lck mRNA was detected in 90% (9/10) of cell lines derived from this tumor. The available data thus suggests that aberrent expression in some cell lineages of lck is associated with invasive or metastatic disease.

Current studies are aimed at discovering the function of lck in normal, dividing T-cells and in the natural history of colon carcinoma and disorders of T-cell growth. This will require a description of the normal control mechanisms of lck transcription and an understanding of the mechanism of release of this control in certain tumors. As such, we are in the process of cloning and characterizing the lck promoter and its upstream control sequences. We have recently isolated rabbit antisera directed against human lck and are now able to study function of the protein. Preliminary data shows that there are at least two forms of the lck protein, one of which is enriched in tumor. Interconversion of these forms seems to be under the control of protein kinase C.

In order to get at the question of the biological function of lck expression we are in the process of attempting to correlate it with other factors implicated in the control of the metastatic phenotype in carcinomas. At the same time, we are in the process of using transfection techniques to express lck in cell lines derived from primary colon carcinoma in an attempt to confer some aspects of the metastatic phenotype. Finally, an important effort will be the develoment of in situ hybridization techniques for this and other c-src family genes in order to assay their expression in detail in clinical specimens and determine their association with clinical disease progression.

C. Inhibitors of c-src family genes. Drugs which could specifically inhibit either expression or protein kinase activity of one of the c-src family genes would have a variety of important uses. Selective inhibition of individual members of this family would allow both an evaluation of their contribution to the neoplastic phenotype and of their specific functions compared to other members of the family. If experiments show that these genes contribute to some aspects of the tumor phenotype, such drugs would have potential therapeutic value.

We have identified four classes of potential agents over the past year and we are now exploring their efficacy and specificity. These include the antibiotics geldanamycin and herbimycin, antisense expression vectors, and

antisense oligonucleiotides, all of which were developed to inhibit protein expression. We are also testing agents developed as competitive inhibitors of tyrosine kinases including erbstatin and analogs thereof, and competitive peptide substrates.

2. Breast cancer studies.

A. As described above, we are studying the biological role of c-src, c-fyn, and other cellular tyrosine kinases in breast cancer as well as colon cancer. The biologic effects of expression of these genes singly or in combination are unknown, as are the potential differences in their functions. It is interesting that while in colon cancer, c-src and c-fyn are uniformly aberrently regulated and lck is activated only in cell lines derived from metastases, in breast cancer cell lines expression of c-src varies directly with estrogen receptor status and inversely with c-fyn expression. None of the breast cell lines overexpress lck. The significance of these observations is being pursued as described above for colon cancer.

B. Expression and function of IGF-1 and IGF-2 in human breast cancer. Our lab is interested in the interaction of activated growth factor receptor kinases and other tyrosine kinases in human breast cancer. We feel selective inhibition of the expression of specific growth factors or their receptors using antisense methodologies will be particularly useful in determining a hierarchy of importance for these factors.

In this regard we are focusing on the study of the insulin-like growth factors in this disease, in collaboration with Dr. Lippman. IGF-1 is mitogenic for human breast cancer cell lines. We have found that these lines also secrete a factor that cross-reacts with IGF-1 in a radioimmunoassay. Northern blots of breast cancer cell line mRNA hybridized to a human IGF-1 probe yield bands of several sizes. However, mRNA mapping in a ribonuclease-protection assay shows that none of the cell lines makes authentic IGF-1 mRNA. We conclude from our data that breast cancer cell lines secrete a tumor specific IGF-1-like molecule. Studies are underway to clone the gene encoding this factor and to purify and characterize the protein.

IGF-2 has also been shown to stimulate breast cancer cell line growth rate. Normally, this factor is predominantly made in cells of mesenchymal origin. In contrast to IGF-1 we have found that a few breast cancer cell lines and many tumor specimens make authentic IGF-2. This project is in its early stages. At present, we are studying the pattern of IGF-2 mRNA expression in normal and malignant tissue and cells and its hormonal regulation. We have synthesized IGF-2 peptides with which we are trying to make specific anti-human IgF-2 antibodies so as to be able to study the protein. The goal is to establish whether IGF-2 is a factor that helps maintain the neoplastic phenotype in breast cancer cells by either an autocrine or paracrine route. To this end, we have begun to do IGF-2 <u>in situ</u> hybridizations on normal and malignant breast tissue.

Relevant Recent Publications:

1) Rosen, N., Bolen, J., Schwartz, A.M., Cohen, P., DeSeau, V., and Israel, M.A. Analysis of pp60^{c-src} protein kinase activity in human tumor cell line and tissues. J. Biol. Chem. 263: 13754-13759, 1986.

2) Bolen, J.B., Veillette, A., Schwartz, A.M., DeSeau, V., and Rosen, N.: Activation of pp60^{c-src} protein kinase activity in human colon carcinoma. Proc. Natl. Acad. Sci. 84: 2251-2255, 1987.

3) Bolen, J.B., Veillette, A., Schwartz, A.M., DeSeau, V., and Rosen, N.: Analysis of pp60^{c-src} in human colon carcinoma and normal colon mucosal cells. Oncogene Res., In Press.

4) O'Shaughnessy, J., DeSeau, V., Amini, S., Rosen, N., and Bolen, J.B.: Increased abundance of the c-src gene product correlates with elevated pp60^{c-src} kinase activity in human neuroblastoma cells. Oncogene, In Press.

5) Lippman, M.E., Dickson, R.B., Gelmann, E.P., Rosen, N., Kaufman, D., Knabbe, C., Bates, S., Kasid, A., Salomon, D., Bronzert, D., and Huff, K.: Growth regulation of normal and malignant mammary epithelium. Cance Res., In Press.

6. Lippman, M.E., Dickson, R.B., Gelmann, E.P., Rosen, N., Knabbe, C., Bates, S., Bronzert, D., Huff, K., and Kasid, A.: Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J. Cellular Biochem., In Press.

7. DeSeau, V., Rosen, N., and Bolen, J.B.: Analysis of pp60^{c-src} tyrosine kinase activity and phosphotyrosyl phosphatase activity in human colon carcinoma and normal human colon mucosal cells. J. Cellular Biochem., In Press.

8. Dickson, R.B., Rosen, N., Gelmann, E.P., and Lippman, M.E.: Oncogenes and breast cancer: importance of receptor- and signal transduction-related protooncogenes. Trends in Pharmacological Sciences, In Press.

9. Veillette, A., Foss, F.M., Sausville, E., Bolen, J.B., and Rosen, N.: Expression of the lck tyrosine kinase in human colon carcinoma and other non-lymphoid human tumor cell lines. Oncogene Res., In Press.

Selected Recent Abstracts:

1. Rosen, N., Bolen, J.B., Biedler, J.L., Thiele, C.J. and Israel, M.A.: Differentiation and transformation in human neuroblastoma: association with pp60^{c-src} activation and N-myc expression. J. Cell. Biochem. Supplement 10C, 122, 1986.

2. Lippman, M., Dickson, R.B., Gelmann, E.P., Knabbe, C., Rosen, N., Valverius, E., Bronzert, D., Bates, S., and Swain, S.: Growth regulation of human breast cancer by secreted growth factors. J. Cell. Biochem. Supplement 11A, 12, 1987.

3. Rosen, N., and Bolen, J.B.: Activation of the $pp60^{c-src}$ protooncogene in human cells. J. Cell. Biochem. Supplement 11A, 17, 1987.

4. Rosen, N., Veillette, A., Foss, F., Yee, D., Schwartz, A.M., O'Shaughnessy, J., Lippman, M.E., and Bolen, J.B.: Regulation of src-related protooncogenes in human carcinoma. Third International Conference on Progress in Cancer Research, San Remo, Italy, 1987.

5. Rosen, N., Yee, D., Foss, F., Kaufman, D., Lippman, M., Schwartz, A., O'Shaughnessy, J. and Bolen, J.B. Overexpression of $pp60^{c-src}$ in human breast carcinoma. Third Annual Meeting on Oncogenes, Frederick, 1987.

6. Veillette, A., Fischer, S., Bolen, J.B., and Rosen, N.: The tyrosine kinase gene lck is expressed in non-lymphoid human tumor cell lines. Third Annual Meeting on Oncogenes, Frederick, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06713-01 M

PERIOD COVERED: October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Multidrug Resistance and Cisplatin Resistance

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

PI:	Tito Fojo	Sr. Investigator	M	NCI
Other:	Robert C. Young	Chief	M	NCI
	Robert F. Ozols	Sr. Investigator	M	NCI
	Lyn Mickley	Med Technologist	M	NCI
	Mace Rothenberg	Med Staff Fellow	M	NCI
	Tom Hamilton	Sr. Staff Fellow	M	NCI

COOPERATING UNITS (if any)
NA

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Medicine Branch

SECTION
Experimental Therapeutics

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The focus of the laboratory is on mechanisms of drug resistance. Two systems are being studied in depth: mechanisms of multidrug resistance and mechanisms of cisplatin resistance. Our aim is to identify the various mechanisms involved in these two systems, by utilizing drug resistant cell lines and various molecular approaches. The availability of probes which can recognize amplified/overexpressed sequences in drug resistant cell lines will offer a starting point for identification of the mechanism involved as well as help in devising approaches to overcome this. In addition, it will provide useful tools to probe primary and recurrent tumor samples to aid in the understanding of the mechanisms of primary and acquired drug resistance in human tumors.

1. Multidrug Resistance
   (a) Identification of tumors expressing mdr1 and attempt to establish clinical correlations.
   (b) Protocols designed to test the significance of mdr1 in a clinical setting. A colon protocol utilizing quinidine as an adjunct to adriamycin and VP-16 is in its final stages. Additional protocols are planned for adrenocortical carcinomas and renal cell carcinomas.
   (c) Development of a nude mouse model of intraperitoneal carcinomatosis with an adenocarcinoma of the colon to test the utility of agents which increase drug resistance in vitro in this model.
   (d) In situ hybridization studies of human tissues and human tumors to identify the sites of expression of mdr1 and its tumor heterogeneity.
   (e) Development of additional cell lines that do not express mdr1, but exhibit drug resistance. This is being done primarily utilizing the breast cancer cell line MCF-7 and selecting with adriamycin. Several clones are currently being characterized.

2. Cisplatin Resistance

   (a) Molecular cloning of amplified sequences from cisplatin resistant cell lines which have been demonstrated to have amplified over 100 kilobases of genomic DNA.
   (b) Differential cDNA screening of libraries from the above cell lines as well as additional cell lines which appear to have different mechanisms of resistance.
   (c) DNA mediated transfection experiments with the goal of confirming the other molecular approaches and also to provide additional ways of cloning the sequences of interest.
   (d) Utilization of specific probes to evaluate the role of metallothionine and DNA polymerase in cisplatin resistance.
   (e) Investigations designed to evaluate the possible utility of metallothionine as a tool for conferring reversible cisplatin resistance to human bone marrow.

3. Resistance in Acute Lymphoblastic Leukemia
   (a) Study of patient samples from both untreated and treated patients to evaluate the significance of mdr1 and DHFR in clinical drug resistance in ALL.
   (b) In situ studies to assess heterogeneity and establish the feasibility of this approach in screening of a large number of small samples.

SELECTED REFERENCES

1. Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M., and Pastan, I.: Expression of a multidrug resistance gene in human tumors and tissues. Proc. Natl. Acad. Sci. USA 84: 265-269, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06714-01 M

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Adoptive Cellular Immunotherapy of Cancer

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

PI:	Michael A. Bookman	Senior Staff Fellow	M	NCI
Other:	Eva Horak	Medical Technologist	M	NCI
	Danny Dean	Biologist	M	NCI

COOPERATING UNITS *(if any)*

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Bureau of Biologics, FDA
Biological Response Modifiers Program, NCI

LAB/BRANCH

Medicine Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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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.)*

Adoptive Chemoimmunotherapy of Murine Leukemia. Cytolytic T lymphocyte (CTL) and helper T lymphocyte (HTL) clones have been established from tumor-bearing mice and evaluated in models of adoptive chemoimmunotherapy (ACIT) of syngeneic murine leukemia (RBL5). Stable antigen-specific clones have been utilized as efficient tools for the investigation of factors important in mediating ACIT of established tumors. We hope to initiate human studies based on data obtained from the murine experimental models.

Current Projects:

1. Characterization of the cellular immune response to retrovirus-induced leukemia.
Tumor immunization promotes host HTL, CTL and suppressor cell immune responses. The majority of HTL are specific for the viral envelope glycoprotein gp70 while CTL recognize other tumor associated antigens (Ags). Non-activated HTL produce interferon- (IFN-) and interleukin-2 (IL-2) after Ag stimulation, but are not directly cytolytic for tumor cells. Recent studies have shown that Ag-stimulated HTL transiently acquire the ability to lyse tumor cells in vitro.

2. Activation, dissemination, and survival of HTL after adoptive transfer.
HTL can be activated in situ to elicit a delayed-type hypersensitivity (DTH) reaction after local transfer with intact tumor cells. The intensity of DTH is sufficient to prevent tumor outgrowth without addition of exogenous factors. HTL can survive and expand in number after adoptive transfer if exogenous IL-2 and Ag are provided. Withdrawal of IL-2 is then associated with rapid reduction in cell numbers. Elimination of

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HTL may partially be the result of an active host response. HTL remain activated after transfer, and can be distinguished from normal host lymphocytes on the basis of size and surface markers. Although HTL can enter lymphoid organs from the bloodstream, their capacity for normal recirculation is markedly reduced. The role of host cells in the elimination of transferred HTL is being studied by selective depletion of host effectors.

3. Activity of HTL and CTL in ACIT. Syngeneic mice with established tumors received therapy with cyclophosphamide (CY) and transfer of clones. ACIT was not effective without CY. Transfer of HTL and CTL prolonged median survival and achieved long-term cures. Combinations of HTL+CTL and HTL1+HTL2 appear synergistic. Control HTL specific for non-tumor Ags are not effective in ACIT and may actually interfere with anti-tumor responses. Exogenous IL-2 is not necessary for successful ACIT.

4. Augmentation of ACIT. Activation of MHC Class-II restricted HTL requires Ag processing by host cells. Pre-activation of host macrophages may enhance HTL anti-tumor responses. IFN-γ and muramyl tripeptide are currently undergoing evaluation in vivo using this model. Monoclonal antibodies, immunotoxins, and heteroaggregated antibodies are also being tested in vitro for their role as potential enhancers of ACIT.

5. Mechanisms of tumor resistance to ACIT. Multiple resistant tumors have been isolated from mice not cured following ACIT. Preliminary data indicate that a high frequency of resistant tumors express an altered form of the gp70 Ag, and are not capable of activating HTL clones.

Abridged References:

Bookman MA, Swerdlow R, Matis LA. (in press, 1987). Adoptive chemoimunotherapy of murine leukemia with helper T lymphocyte clones. J Immunol

Bookman MA, Groves ES, Matis LA. (in press, 1987) Adoptive chemoimmunotherapy of murine leukemia: dissemination, survival, and function of helper T lymphocyte clones. In: Cellular Immunotherapy of Cancer. Eds: Truitt RL, Gale RP, Bortin MM. Alan R. Liss, Inc., New York, NY.

Bookman MA, Groves ES, Matis LM. 1986. Expression of MEL-14 antigen is not an absolute requirement for dissemination to lymph nodes after adoptive transfer of murine T lymphocyte clones. J Immunol 137:2107-2114.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM03024-18 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Treatment of Extensive Stage Small Cell Lung Cancer

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

PI:	Daniel C. Ihde, M.D.	Senior Investigator	NCI-NMOB
Others:	Adi F. Gazdar, M.D.	Senior Investigator	NCI-NMOB
	R. Ilona Linnoila, M.D.	Senior Investigator	NCI-NMOB
	Stephen R. Veach, M.D.	Head, Hematology/Oncology Branch	NHBETH
	John D. Minna, M.D.	Chief, NCI-NMOB	NCI-NMOB
	Herbert K. Oie, Ph.D.	Microbiologist	NCI-NMOB
	Edward K. Russell	Chemist	NCI-NMOB

COOPERATING UNITS *(if any)*

Radiation Oncology Branch; Biostatistics & Data Management Section; Surgical Oncology Division, Naval Hospital Bethesda; Hematopathology Branch, Naval Hospital Bethesda

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Clinical Investigations

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	2	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.)*

Although a dose-response curve clearly exists for alkylating agents in the initial chemotherapy of small cell lung cancer, the therapeutic benefit of higher than standard doses of the more recently introduced regimen of etoposide/cisplatin (VP16/PLAT) is uncertain. We randomized at least partially ambulatory patients with extensive stage SCLC and without major organ dysfunction to receive either VP16 80 mg/m squared + PLAT 27 mg/m squared Days 1-5 q 3 wks or VP16 80 mg/m squared Days 1-3 + PLAT 80 mg/m squared Day 1 q 3 wks for the first 6 wks of therapy. Nonambulatory patients and those with organ dysfunction were assigned standard dose treatment. All patients received the standard dose regimen during Wks 7-12. From Wks 13-24, patients in complete response (CR) continued standard dose VP16/PLAT, while all other patients received a new 3-drug regimen that led to further improvement in response in only 4 cases. CR's were given prophylactic cranial irradiation. Sixty-three patients have been entered (46 of whom were randomized). With a median follow-up of 18 mos, preliminary results are:

	N	CR	CR+PR	Med Surv	Nadir WBC	Nadir Plt
High	21	29%	86%	13 mos	1,200	47,000
Standard	25	32%	88%	11 mos	3,200	196,000
Nonrad	17	6%	71%	5 mos	2,000	124,000

CR rates (p=0.99) and survival (p=0.83) were similar in patients randomized to high and standard dose therapy. There were 2 treatment-related deaths in the high and none in the standard dose arm. We conclude 1) standard dose VP16/PLAT is at least as active as any regimen we have ever utilized for extensive stage SCLC and produces only modes myelotoxicity, and 2) there is no evidence of superior efficacy when drug doses are increased by 67% during the first 6 wks.

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Treatment of Extensive Stage Small Cell Lung Cancer

Professional Staff:

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	Stephen R. Veach, M.D.		NCI-NMOB
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	Edward K. Russell		NCI-NMOB

II. Collaborating Branches

a) Radiation Oncology Branch:
Eli Glatstein, M.D.; Jane Grayson, M.D.

b) Biostatistics and Data Management Section:
Seth M. Steinberg, Ph.D.

c) Surgical Oncology Division, Naval Hospital Bethesda:
Bimal C. Ghosh, M.D.

d) Hematopathology Branch, Naval Hospital Bethesda:
James Cotelingham, M.D.

Objectives, Rationale, and Background:

This trial has several objectives. We wished to determine in a prospective randomized fashion whether high doses of etoposide (VP16) and cisplatin (PLAT) given during a six-week induction period would produce higher complete response rates and better survival than standard doses of the same drugs in patients with extensive stage small cell lung cancer (SCLC). We also wished to assess the feasibility and value of individualized chemotherapy selection based upon in vitro drug testing of tumor cell lines derived from pre-treatment patient tumor specimens. Objectives of this portion of the study were to determine the fre quency with which tumor-containing specimens can be obtained from unselected patients with extensive stage SCLC, the frequency of successful cell culture and drug sensitivity testing, the degree of heterogeneity of drug sensitivity among different cell lines, the correlation between in vitro drug sensitivity and clinical response, and the clinical utility of individualized drug selection based upon in vitro data.

The introduction of combination chemotherapy into the management of SCLC has led to four- to five-fold improvement in median survival and five-year disease-free survival in a small fraction of patients. Although median survival is improved to approximately the same degree compared to untreated patients in limited stage and extensive stage disease, survival of two years or more only

rarely occurs in patients with extensive disease, defined as tumor extending beyond the hemithorax of origin and the regional lymph nodes. Furthermore, chest irradiation has never been suggested to yield any survival benefit in extensive stage patients. Therefore, virtually all patients with extensive SCLC are suitable subjects for investigational chemotherapy studies.

Methods Employed

Moderately aggressive chemotherapy which produces leukopenia in the range of 1,000/mcl has been shown to be superior to less intensive treatment that is virtually never associated with leukopenic fever in both randomized and non-randomized studies (1). However, even more intensive initial (or induction) therapy which is so myelosuppressive that hospitalization of all patients is required has not been demonstrated to provide additional benefit, although randomized studies have not addressed this issue. In most of these studies, the drugs given in very high doses have been restricted to cyclophosphamide, doxorubicin, and VP16. VP16/PLAT has been shown to be a highly synergistic combination regimen in treatment of murine leukemia and in early studies appears to be as active as most three- or four-drug combinations in patients with SCLC. VP16/PLAT is also more active than VP16 alone as a salvage regimen in this tumor. PLAT in higher than conventional doses appears to have increased activity in testicular and perhaps ovarian cancer. Although higher than standard doses of VP16/PLAT have been employed in small studies in SCLC, the issue of dose-response with this combination has not been addressed in a prospective randomized trial. We therefore initiated such a study. The first four patients randomized to the high dose regimen received VP16 120mg/m^2 x 5 and PLAT 40mg/m^2 x 5. Two died of infection before Day 21 without recovery from myelosuppression, and the doses of drugs on the high dose arm were subsequently reduced to VP16 80mg/m^2 x 5 and PLAT 27mg/m^2 x 5. Throughout the trial, doses on the standard arm have been VP16 80mg/m^2 x 3 and PLAT 80mg/m^2 x 1. Since a significant minority of extensive stage SCLC patients are not candidates for a very myelosuppressive regimens, such patients (deemed "poor risk") are not randomized but rather assigned to standard dose therapy.

For the past 10 years, the human tumor stem cell assay of Hamburger and Salmon has been most commonly employed for in vitro drug testing of human cancer. In applying this test to fresh tumor specimens from our SCLC patients, however, we found that sufficient tumor colonies for adequate in vitro testing of even a single drug were present only 23% of the time. Clearly, different approaches were needed to apply in vitro drug testing to a large fraction of patients. Since our laboratory has considerable experience in establishing permanent cell lines of SCLC, we decided to utilize cell lines rather than fresh tumors for drug testing. Compared to fresh tumors, cell lines provide tumor cells that are free of contaminating stromal cells and can be subjected to repeated testing. The time from specimen procurement to assay results, however, is delayed.

A modification of the Weisenthal dye exclusion assay was employed for drug testing because the assay is technically simple, does not require a single cell suspension, can be completed in four days, and can be applied to many tumors and most cell lines. Reading the assay, however is labor intensive and subjective and can be confounded by cell clumping.

Major Findings

Sixty three patients have been entered. Median follow-up from time of patient entry is approximately 20 months. Seventeen of the 63 patients were assigned standard dose therapy because of poor performance status, brain, lung, or cardiac dysfunction, or refusal to be randomized. The remaining 46 were randomized to receive high or standard dose VP16/PLAT for the first 6 weeks of therapy.

On the high dose arm, 18 (86%) of 21 have responded to therapy, including 6 (29%) complete responders, and actuarial median survival is 13 months. On the standard dose arm, 22 (88%) of 25 patients responded, including 8 (32%) complete responders, and actuarial median survival is 11 months. There is no significant difference between the two groups in complete response rate ($p = 0.99$) or overall survival by the logrank test ($p = 0.83$). As expected, the response rate (6% complete, 65% complete plus partial) and survival (actuarial median 6 months) are inferior in patients judged not suitable for randomization. Among all 63 patients, performance status and number of distant org n systems involved with metastatic disease (0-2 vs. 3-7) are highly significant predictors of survival ($p < 0.001$ and $p = 0.004$, respectively).

Hematologic toxicity is significantly worse on the high dose induction program (median nadir WBC count 1,200/mcl and platelet count 47,000/mcl) compared with the standard dose induction (median nadirs 3,200 and 196,000, respectively). Among the poor risk nonrandomized patients, median nadir WBC count has been 2,000/mcl and median nadir platelet count, 124,000/mcl. There have been four treatment-related deaths, all due to myelosuppression and infection, two on the high dose arm prior to lowering of the drug doses and two in poor risk patients assigned standard dose therapy. Although only 25 patients have been treated, the standard dose regimen yields results at least as good as our historical experience in good risk extensive stage SCLC with considerably less hematologic toxicity, suggesting it may have a superior therapeutic index.

A total of 168 pre-treatment staging specimens have been submitted for cell culture from these 63 patients (2.7/patient). Sixty-nine specimens (41%) contained tumor cells. Twenty-two cell lines, defined as sufficient in vitro amplification of tumor cell number to allow testing of multiple drugs in duplicate at three concentrations, have been obtained. The largest numbers of positive specimens and cell lines were derived from bone marrow, peripheral lymph nodes, and pleural effusions. Procurement of only five specimens required administration of general anesthesia, but three of these five procedures were performed for diagnostic purposes. Among the 63 patients, at least one staging specimen reached the cell biology laboratory in 62 (98%), and a tumor-containing specimen was procured from 46 (73%) of these previously untreated patients. A cell line was obtained from 21 (33%), or 46% of patients from whom a tumor-containing specimen was available. In addition, tumor-containing specimens have been obtained from 12 of these patients after tumor progression on chemotherapy, and a cell line has been successfully grown from five.

Actuarial median survival of patients from whom a tumor cell line was successfully grown, patients from whom a tumor-containing spec men was obtained but did not grow in vitro, and patients from whom no tumor-containing specimen could

be procured was 7, 11, and 17 months respectively.. Patients with no tumor specimen had superior survival by the logrank test ($p < 0.02$). The survival of patients whose tumor specimens were or were not successfully cultured was not significantly different ($p = 0.55$). Thus, whether a patient had sufficient tumor dissemination that a biopsy specimen could be relatively easily obtained was of greater prognostic import than whether a cell line could be established from a positive biopsy specimen.

In vitro drug testing has been completed on tumor cell lines derived from 21 previously untreated SCLC patients. Seven drugs known to be active against SCLC in vivo were tested. These included VP16, PLAT, doxorubicin, mechlorethamine (a substitute for cyclophosphamide), vincristine, lomustine, and methotrexate. The relative activity of the drugs was ranked on the basis of per cent cell survival at arbitrary reference concentrations for each drug as modified from Weisenthal. The frequency with which each drug was one of the three most active drugs tested at its reference concentration ranged from 14/21 for VP16 to 4/20 for methotrexate. In these patients, there were 14 different three-drug regimens selected as the "in vitro best regimen," and no three-drug regimen was selected in more than two patients. Thus, a considerable degree of heterogeneity in drug sensitivity exists among cell lines from untreated patients. This result is important, since if all SCLC cell lines had homogeneous drug sensitivity patterns, no individualized drug selection for a specific patient would be possible.

In vitro drug sensitivity of these cell lines correlated extremely well with response to therapy to VP16/PLAT. In 14/15 (93%) lines from patients with complete or partial response at 12-week restaging, two or more drugs were "active," defined as < 50% cell survival at the reference concentration, and 43/96 (45%) individual drug assays were "active." Sensitivity patterns were strikingly different in the four lines from patients who never responded to VP16/PLAT or had progressed by Week 12. In none of these lines were two or more "active" drugs identified, and only 1/25 (4%) of individual drug assays indicated "activity." P values for these differences were 0.003 and 0.0001, respectively. For each of the seven drugs considered individually, lines from responding patients always exhibited a lower mean cell survival at the reference concentration than lines from non-responding patients. Evaluation of these differences with the 2-sample rank test, which does not depend upon an arbitrary cutoff point to define "activity," yielded p values of less than 0.05 for VP16, doxorubicin and mechlorethamine, and less than 0.15 for PLAT, vincristine, and methotrexate.

Complete response rates to the first chemotherapy regimen given after VP16/PLAT were compared in patients receiving an "in vitro best regimen" based on in vitro drug testing, or in those receiving vincristine/doxorubicin/cyclophosphamide (VAC) when in vitro drug testing results were not available for whatever reason. Twenty-five patients were treated with VAC after failure to achieve complete response by Week 13, and five after relapse from complete response induced by VP16/PLAT. In these 30 patients, there were two complete responses (7%). Among the 12 patients who received their "in vitro best regimen," 10 had failed to achieve complete response at Week 13, and two had relapsed. Three patients (25%) attained complete response to their chemotherapy program based on in vitro drug testing.

Significance to Biomedical Research and the Program of the Institute

Thus far, there is no indication from this study that a high dose regimen of VP16/PLAT (67% higher doses of each drug) is in any way superior to standard doses of this two-drug regimen. On the other hand, the standard dose program is well tolerated and may be as effective as any other SCLC regimen, based on this data and that of others. Given the low complete reponse rate to any of the drug programs given to partial or non-responders at Week 13, it is likely that most or all of the survival benefit our patients received from therapy was produced solely by VP16/PLAT.

The interim results of this trial serve to emphasize several problems that arise in implementing a program of individualized chemotherapy selection with our current technology and study design. First, procurement of tumor specimens, establishment of cell lines, and drug testing are extremely labor intensive and time consuming. More efficient assay techniques and better understanding of the relationship between in vitro and in vivo pharmacokinetics would be valuable. Within the next few months, we intend to implement a tetrazolium-based semiautomated colorimetric assay for drug testing in this study whenever possible. This assay is both objective and rapidly read. Second, drug testing has been possible in only one-third of patients, and improved methods of cell culture are still needed. We believe these interim results justify the more frequent employment of major surgical procedures to procure larger, more rapidly grown tumor specimens in good risk consenting patients, and have already begun such a program in limited stage patients, who would be expected to more frequently be able to tolerate elective general anesthesia. And third, with the time required to establish and perform drug testing on cell lines, treatment based on in vitro testing can often be given only 10 to 12 weeks after a tumor specimen is obtained and may not be relevant to the in vivo drug sensitivity pattern present in residual tumor cells present at that time. Procurement of larger tumor specimens could help to alleviate this problem and allow more rapid drug testing and quicker administration of "individualized" chemotherapy.

Proposed Course

A statistical analysis done six months ago, when 37 patients had been randomized, 95% confidence limits for differences in 12-month survival ranged from favoring the high dose arm by as much as 46% to favoring the low dose arm by as much as 22%. At that point, we elected to enter an additional 10 patients on each arm, since if the observed complete response rates in the additional patients remained at 30% for each arm, a doubling of complete response rates from 25% to 50% with high dose therapy could be excluded with more than 95% confidence. The study will thus be analyzed for purposes of deciding whether to continue randomization when 11 more patients suitable for randomization are accrued.

Despite these problems and the preliminary nature of our results, we believe several conclusions are justified. First, results of drug sensitivity testing of tumor cell lines are highly correlated with response to initial chemotherapy. Second, preliminary results utilizing in vitro drug testing for individualized selection of chemotherapy regimens suggest modest potential for therapeutic benefit. Third, the close correspondence between in vitro and in vivo response to drugs provides justification for the use of human cancer cell lines in

screening for new chemotherapeutic agents. And finally, the availability of multiple SCLC tumor cell lines from patients whose clinical course is well characterized, including some paired lines from patients before and after in vivo chemotherapy, may prove useful in helping to elucidate the basis for drug resistance and other biologic properties of this tumor.

Publications

1. Ihde, D.C., Russell, E.K., Oie, H.K., Linnoila, R.I., Steinberg, S.M., Gosh, B.C., Schumacher, H.R., Minna, J.D., Gazdar, A.F. Prospective clinical trial of individualized chemotherapy based on in vitro drug sensitivity testing in extensive stage small cell lung cancer. In, Adjuvant Therapy of Cancer V, Salmon S.E. (ed.). Orlando, Grune & Stratton, in press.

2. Ihde, D.C., Johnson, B.E., Mulshine, J.L., Sausville, E.A., Veach, S.R., Steinberg, S.M., Edison, M., Lesar, M., Minna, J.D. Randomized trial of high dose versus standard dose etoposide and cisplatin in extensive stage small cell lung cancer (abstr). Proc. Am. Soc. Clin. Oncol. 6: 181, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06575-12 NMOB

PERIOD COVERED

September 30, 1986 to October 1, 1987

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

Oncogenes, Chromosomal Deletions & Growth Factors in the Pathogeneis of Lung Cancer

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

PI:	John D. Minna, M.D.	Branch Chief	NCI-NMOB
Others:	Marion Nau	Chemist	NCI-NMOB
	Joseph Fedorko	Microbiologist	NCI-NMOB
	Michael Birrer, M.D.	Medical Staff Fellow	NCI-NMOB
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Susan Naylor, Ph.D., Univ. Texas San Antonio; Berton Zbar, M.D. FCRC; Jacquline Whang-Peng, Medicine Branch, NCI

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Molecular, Genetics, and Immunology

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	10	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.)*

Nuclear acting cellular proto-oncogene DNA status and mRNA expression has been characterized in a large number of lung cancer cell lines. These include c-myc, N-myc, L-myc, and p53. A variety of DNA changes have been found including amplication and gene rearrangements. A complex pattern of expression has been found with high levels of expression seen without gene amplification. The structure of the L-myc proto-oncogene, first described by our Branch has been worked out including the nucleotide sequence of the normal genomic L-myc gene as well as the cDNA sequence. This has led to the identification of a complex pattern of alternative mRNA splicing leading to a variety of L-myc mRNAs. The proteins coded for by the L-myc gene have been identified and partially characterized.

The 3p(14-23) deletion first found cytogenetically has been shown to be a true DNA deletion by restriction fragment length polymorphism analysis. This has been seen in both small cell and non-small cell lung cancer making it the most frequent deletion in a common solid tumor.

A series of peptides produced by lung cancer cells including insulin-like and transferrin-like growth factors, and opioid peptides have been identified as being new candidates for autocrine growth factors in the pathogenesis of lung cancer.

(cont'd)

Others:	Jacques DeGreves, M.D.	Fogarty Fellow	NCI-NMOB
	Joachim Schuette, M.D.	EORTC Fellow	NCI-NMOB
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	Rhoda Maneckjee, Ph.D.	Guest Researcher	NCI-NMOB
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	Bruce Johnson, M.D.	Senior Investigator	NCI-NMOB
	Edward Sausville, M.D., Ph.D.	Senior Investigator	NCI-NMOB
	James Battey, M.D., Ph.D.	Senior Investigator	NCI-NMOB
	Adi Gazdar, M.D.	Chief, Human Tumor Biology Sec.	NCI-NMOB

PROJECT DESCRIPTION

Oncogenes, Chromosomal Deletions, and Autocrine Growth Factors in the Pathogenesis of Lung Cancer

Principle Invest.:	John D. Minna, M.D.	Branch Chief	NCI-NMOB
Others:	Marion Nau	Chemist	"
	Joseph Fedorko	Microbiologist	"
	Michael Birrer, M.D.	Medical Staff Fellow	"
	Frederick Kaye, M.D.	Medical Staff Fellow	"
	Eric Seifter, M.D.	Medical Staff Fellow	"
	Jacques DeGreves	Fogarty Fellow	"
	Joachim Schuette, M.D.	EORTC Fellow	"
	Frank Cuttitta, PhD	USUHS Assist. Prof.	"
	Phil Kasprzyk, PhD	Hybritech Fellow	"
	Rhoda Maneckejee, PhD	Mathers Fellow	"
	Michelle Vinocour, PhD	Mathers Fellow	"
	Bruce Johnson, M.D.	Investigator	"
	Edward Sausville, M.D., PhD	Investigator	"
	James Battey, M.D., PhD	Investigator	"
	Adi Gazdar, M.D.	Section Chief	"

Genetic Changes Involved in the Pathogenesis of Human Lung Cancer Including Oncogene Activation, Chromosomal Deletions, and Autocrine Growth Factor Production

Principal Investigator: John D. Minna, M.D.

A. Objectives

Overall Objective:

To identify and characterize the genetic changes (somatic and constitutional) leading to the pathogenesis of lung cancer.

Project #1

Role of Cellular Proto-oncogenes in the Pathogenesis of Lung Cancer

1. To identify the nuclear acting proto-oncogenes expressed in lung cancer cells (c-myc, N-myc, and L-myc, p53, c-fos, c-myb, and c-jun) including studies of tumor cell lines and clinically relevant biopsy samples of tumors obtained directly from patients.
2. To relate the expression of the nuclear acting proto-oncogenes to the development and progression of lung cancer including potential relationships to chromosomal deletions and autocrine growth factor production.
3. To characterize the expression of the nuclear acting proto-oncogenes during normal fetal lung development and relate these patterns of expression to those found in tumors.
4. To identify DNA changes and other regulatory mechanisms specifically associated with the expression of these genes.
5. To understand the genomic structure and sequence of the L-myc gene and its various message forms, including promoter and other regulatory sequences, and compare these to the analogous regions for c-myc and N-myc.
6. To characterize the protein products of the various L-myc mRNAs including their biologic activity, cellular localization, half life and potential function.
7. To identify other members of the myc family of genes and determine if they are expressed or genetically altered in lung cancer.
8. To identify other known cellular proto-oncogenes that are amplified, rearranged, or mutated in lung cancer cells.

Current Working Model of the Pathogenesis of Lung Cancer:

Our results coupled with knowledge of continued carcinogen exposure by cigarette smoking, the findings of oncogenes activated by mutation in human tumors, and the role of proto-oncogene cooperativity in

experimental systems, led to our current working model of the steps involved in the pathogenesis of human lung cancer. These include:

1. Exposure to carcinogens including cigarette smoke (through active or passive routes) and possible evironmental carcinogens with inheritance of a debrisoquine metabolic phenotype giving a genetic predisposition to carcinogen metabolism.

2. Possible inheritance of a recessive gene in chromosome region 3p(14-23).

3. Induction of GRP secretion by isolated neuroendocrine cells in the adult lung with subsequent autocrine stimulation of these normal neuroendocrine cells via activation of L-myc and clonal proliferation of the neuroendocrine cells.

4. Increased GRP production by the expanded population of neuroendocrine cells and further autocrine stimulation as well as paracrine stimulation of neighboring cells by the high levels of GRP. This in turn results in more L-myc activation with multifocal clonal proliferation allowing for the clonal expansion of any mutations in cellular proto-oncogenes induced by the carcinogens.

5. Activation of other growth factor production by the neuroendocrine and other replicating bronchial epithelial cells such as insulin like growth factors possibly through releasing factor activity of GRP directly or indirectly with potentiation of GRP effects by the insulin like activity such that large portions of the bronchial epithelium are now involved in autocrine growth stimulation.

6. Clonal expansion of other genetic changes induced by cigarette smoke carcinogens such as 3p mutations, translocations, or deletions to uncover an inherited recessive lesion in the 3p region or cause lesions in both copies of this chromosome. The large size of the 3p deletion as well as cytogenetic evidence indicating involvment of genes in both the 3p14.2 and 3p23-25 regions, suggests the possiblity that several genes are required for normal growth control and thus indicates a potentially large target size for carcinogen attack in the 3p area.

7. Loss of normal differentiation and growth regulatory signals via the loss of the 3p function leading to formation of carcinoids, adenomas, or carcinoma in situ.

8. Accumulation of other proto-oncogene mutations or activation such as mutations in ras family members, deregulated expression of c- and N-myc to give invasive carcinoma and loss of growth factor regulation.

9. Amplification or further deregulation by other mechanisms to give very high levels of myc family member expression, as well as accumulation of other mutations leading to metastatic cancer.

Progress Report

Minna Project #1:

Expression and DNA Status of Nuclear Acting Proto-Oncogenes in Lung Cancer Cells.

myc family members and p53:

We have studied the expression of c-myc, N-myc, L-myc, p53, c-myb, c-fos, and c-jun in polyA(+) RNA from small cell and non-small cell lung cancer cell lines using standard Northern blot technology. The levels were quantitated by timed autoradiography and densitometer tracings of films and the use of a reference gene (beta actin) mRNA signal. c-myc, L-myc, and p53 were found expressed in many examples of both small cell and non-small cell lung cancer. In contrast, we so far have found N-myc expressed only in small cell lung cancer. We found tumor cell lines expressing relatively high levels of each of these four genes while other lung cancer lines failed to express one or more of the genes at detectable levels. (Vinocour et al. 1987). In addition, some tumors simultaneously expressed several of these genes.

c-myb:

While others (Griffin & Baylin 1985) have reported more frequent expression of c-myb in small cell lung cancer, we have found only one

example of high level expression in a small cell lung cancer line (NCI-H526) which is amplified for c-myb (Nau et al. 1987). Interestingly, line H526 is also amplified and rearranged for N-myc (Nau et al. 1986). Dr. Kuehl is planning to study c-myb expression in this line in detail as part of his long term interest in this gene (see Dr. Kuehl's annual report).

c-fos:

We found c-fos to be constitutively expressed only at low levels in the lung cancer cell lines and have not yet found examples of c-fos amplification or rearrangement. Dr. Sausville's group is studying c-fos expression after growth factor activation in these cells (see Dr. Sausville's annual report).

c-jun:

While the studies on c-jun are in their early phase, we have seen several examples of expression of c-jun in lung cancer using the currently available avian retrovirus probe (kindly provided by Dr. P. Vogt of Univ. Southern Calif.) (Maki et al. 1987). Recently, we have found c-jun amplified 5 to 15 fold in several lung cancer lines and these lines express a mRNA detected by the avian jun probe. Thus, we have frequently found expression of c-myc, N-myc, L-myc, p53, and c-jun in human lung cancer cell lines and infrequent expression of c-myb and c-fos.

Early Clinical and Biologic Correlations of myc family member amplification in small cell lung cancer:

c-myc amplification is associated with decreased patient survival

As indicated above, the initial search for amplified oncogenes was prompted by the presence of DMs and HSRs in the variant small cell lung cancer lines that exhibited more aggressive behavior in vitro (Little et al. 1983; Gazdar et al. 1985b). To try to directly tie this to the clinic, Dr. Bruce Johnson has performed two important experiments (Also see Dr. Johnson's Annual Report). In a retrospective analysis, he correlated patient survival with the presence of c, N, or L-myc amplification in tumor cell lines from extensive stage small cell lung cancer patients and found

signficantly shortened survival in patients whose tumor cell lines demonstrated c-myc amplification (Johnson et al. 1987). This is, of course, similar to the findings of N-myc amplification in human neuroblastoma with shortened patient survival or in the more undifferentiated neuroblast cells (Brodeur et al. 1984; Schwab et al. 1984).

Transfection of a c-myc gene into a small cell lung cancer line alters its morphologic and growth characteristics

The second type of experiment Dr. Johnson performed was to transfect a normal human c-myc gene physically linked to the bacterial neomycin gene and SV 40 promoter (from pSV2 neo) into a small cell lung cancer line not expressing c-myc and to test for alteration in the transfectant phenotypes. He found: 1.) that the morphology in vitro and in xenografts of the tumor cell line expressing the transfected c-myc were altered and similar to those of the c-myc amplified variant tumors and; 2.) that the growth rate and cloning efficiency of the transfectants were increased, again similar to the c-myc amplified lines. In fact, the changes appeared to be proportional to the amount of c-myc mRNA expressed (Johnson et al. 1986a). However, the expression of differentiated markers such as GRP and L-dopa decarboxylase was not altered. While it is possible that higher levels of c-myc expression may alter the expression of these differentiated functions (see Dr. Johnsons' annual report), it was clear that the introduction of a functional c-myc gene altered the morphology and growth characteristics of small cell lung cancer in a manner consistent with this gene's expression playing an important role in the clinical behavior of the tumors.

Expression in tumor samples taken directly from patients:

These early clinical correlations with c-myc amplification and expression have prompted plans for a prospective study of clinico-pathologic correlation of myc family member expression in patients' tumors. The study of expression of these genes in tumor samples taken directly from patients is just beginning and required the development of technology (in situ mRNA hybridization and immunohistochemistry) to detect proto-oncogene mRNA and proteins in clinically available (ie. formalin fixed, paraffin embedded) tumor speci-

mens (Gu et al. 1986 abstract). These studies identified the expression of N-myc in a primary tumor biopsy sample, placing the activation of the myc genes in a tumor before metastasis or cell culture.

DNA status of the genes expressed:

Members of the myc family were found to be expressed with and without associated DNA amplification (Nau et al. 1985; Nau et al. 1986; Vinocour et al. 1987). However, nearly all of the examples of gene amplification were found in small cell lung cancer. Nevertheless, the non-small cell tumors frequently expressed myc mRNA (particularly c-myc) to levels comparable to those seen in small cell lung cancer lines with amplified myc genes, suggesting that non-small cell lung cancer uses a different mechanism than small cell lung cancer in deregulating myc gene expression. Amplfication of p53 or c-fos was not seen in any of our tumor line DNAs despite high levels of expression of p53.

We have found the DNA amplifications not only in cultured tumor cells but also in tumor specimens harvested directly from patients (usually at the time of necropsy). In those patients whose tumors showed oncogene amplifcation and where normal tissue was also available, we have never found amplication or rearrangements of the cellular proto-oncogenes in the normal tissue, showing that these genetic changes are acquired in the tumor (Nau et al. 1986).

DNA Rearrangements and abnormal mRNAs:

Except for uncommon deletions of the p53 gene associated with lack of p53 expression, the only DNA rearrangements for the nuclear acting oncogenes we have seen occurred with gene amplification (Nau et al. 1986; Brooks et al. 1987). However, we have not looked extensively with flanking probes for the various genes in their non-amplified state. In cases where HSRs were found these were almost never at the site of the germ line location of the myc family gene (Whang-Peng et al. 1982b) (8q for c-myc, 2p for N-myc, and 1p for L-myc) suggesting these genes and their amplicons had moved to different chromosomes. Also, several of the amplified, rearranged DNAs have been associated with abnormal mRNA sizes particularly for N-myc and c-myc (Nau et al. 1986). One amplified

and rearranged L-myc DNA of particular interest is discussed below. Thus, in some cases DNA rearrangements and abnormal mRNAs characterize myc family expression in lung cancer cells.

We have seen abnormal p53 mRNA sizes without DNA amplification or rearrangements. In lung cancer cell lines expressing a normal size p53 mRNA, p53 protein was detected by immunohistochemistry in the nucleus of the tumor cells. However, in the particular tumor cell line with both normal and abnormal sized p53 mRNAs, we also noted cytoplasmic staining for p53 suggesting a change in the cellular location of the protein product associated with the abnormal mRNA (Vinocour et al. 1987).

Developmental Expression of L-myc:

In collaboration with Dr. F. Alt's group the developmental and tissue distribution of expression of c-myc, N-myc, and L-myc were examined in newborn and adult mouse tissues (Zimmerman et al 1986). While c-myc was expressed in many different tissues and often in both newborn and adult tissues, N-myc and L-myc had a much more restricted pattern being predominantly expressed in brain, kidney, gut (N-myc), or brain, kidney and lung (L-myc). In most instances N-myc and L-myc were expressed in newborn but not adult tissues. However, L-myc was expressed in both newborn and adult lung albeit at reduced levels in adult lung. Thus, our current working hypothesis is that N-myc and L-myc may provide myc like functions in a more restricted (differentiated) population of cells during development.

Characterization of the L-myc gene:

Because L-myc was expressed in both small cell and non-small cell lung cancer and had interesting developmental expression patterns, we set out to fully characterize the L-myc gene in normal and tumor cell DNA. A considerable portion of our work has focused on defining the genomic and cDNA structure of this gene, its DNA sequence, mRNA forms, patterns of expression, protein product(s), biologic activity and searches for genetic changes in human tumors.

Structure and sequence of L-myc:

Initial strategy for genomic cloning:

The initial clone for L-myc was obtained by size fractionating Eco RI cleaved genomic DNA from the SCLC line H378, which had amplfied one of its L-myc alleles. The amplified Eco RI fragment containing the L-myc gene was cloned in a lambda replacement vector and identified using a homologous N-myc probe. This cloned 10 kb Eco RI fragment was subsequently subcloned into plasmid (pLmyc10) (Nau et al. 1985). This clone in turn was used to identify a unique Eco RI- Sma I fragment which allowed isolation of bacteriophage clones from a human placental genomic DNA library (provided by Dr. P. Leder). These clones were restriction mapped, sub-cloned, compared to genomic DNA from a variety of tumor and normal DNAs, and partially sequenced. This sequencing both identified the gene as containing a region of great nucleotide sequence homology to the second exon of c-myc and N-myc, but also as containing novel sequence indicating it as a different gene (Nau et al. 1985).

Isolation of L-myc cDNA clones:

Since the Eco RI - Sma I fragment was contiguous to the myc homology region, we reasoned it may encode an exon, or part of one, and were able to show that it was expressed in poly A(+) RNA from line H378 (Nau et al. 1985). A series of bacteriophage cDNA libraries were constructed from small cell lung cancer lines expressing L-myc including: 1) an oligo-dT primed lamda gt10 library from NCI-H378 (amplified and rearranged for L-myc see below); 2) an oligo dT primed lambda gt 11 library from NCI-H209 (expressing but not amplified for L-myc); 3) a lambda gt10 library of NCI-H510 (expressing and amplified for L-myc) random primed and primed with specific oligonucleotides to obtain 5' L-myc cDNA clones. From these libraries we have isolated cDNAs representing several different mRNA forms observed in RNA blots (see below). These have been used as probes in RNA and DNA analysis as well as sequenced and used to locate gene boundaries and exons in L-myc genomic DNA clones.

L-myc has a three exon structure and several domains of striking nucleotide and amino acid sequence homology with c-myc and N-myc:

The results so far show that L-myc has an overall topography similar to c-myc (Battey et al. 1983) and N-myc (Stanton et al. 1986; Kohl et al. 1986) with three exons including a non-coding first exon (found in c-myc and N-myc) and a long untranslated region in the third exon (similar to N-myc) (Minna et al. 1986)(Figure 1). There is a long open reading frame beginning with a Met residue at the 5' boundry of the myc family concensus second exon extending for 362 amino acids until a termination codon in the middle of the third exon. There are four major domains of nucleotide and amino acid sequence homology between the three myc genes (Figure 2). L-myc codes for a shorter protein than does c-myc (439 amino acids) and N-myc (464 amino acids). Between the areas of homology the nucleotide sequences of the three genes are quite different. Of interest, the second/ third exon spice boundary appears highly conserved in the three genes. In addition, the conserved domains appear similar to areas reported by others (Lee et al. personal communication) to be required for biologic activity of c-myc.

L-myc undergoes alternative splicing to generate multiple different mRNAs:

Alternative splicing of the intron between the 2nd and 3rd exons:

L-myc has at least three and probably more than four different mRNAs (see Figure 1). There are two major mRNA species at approximately 4 and 2 kb. These different species are explained by alternative splicing of L-myc mRNA to give the 4 kb form with all three exons and the 2 kb form with only the first and second exons. Thus, there is another polyadenylation site in the intron between the second and third exons. cDNA clones for the 2 kb mRNA form have been isolated from all three small cell lung cancer libraries and all have the same polyadenylation site. Thus, the 2 kb form lacks the third exon, an area reported to be necessary for transforming activity of c-myc (Lee et al.

personal communication). However, there is an additional open reading frame in the 2 kb form potentially adding 15 amino acids onto the second exon. Thus, we predict that any protein coded for by the 2 kb mRNA should not only lack the third exon but have a short polypeptide extension of exon two protein sequences (see below).

Alternative splicing between the 1st and 2nd exons:

In addition, cDNA clones have demonstrated another splicing variation including forms with and without the first intron removed. By northern blot analysis, the two forms appear to be equally represented in polyA(+) mRNA. Thus, it appears the 4 and the 2 kb form can each be subdivided into a type with and without a longer first exon giving four different mRNAs from the L-myc gene.

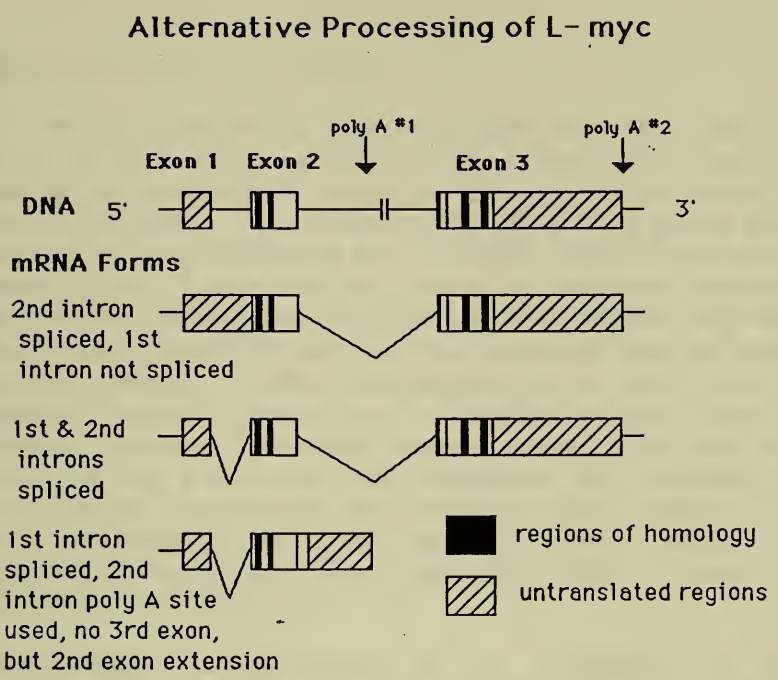

Figure 1

Rearranged forms of L-myc in Small Cell Lung Cancer:

Our analysis of placental genomic L-myc clones revealed that the first tumor DNA (H378) used to isolate L-myc had a rearrangement of the amplified allele at its 5' end. With determination of the normal L-myc structure we have shown that this is an inversion/ deletion in the first exon area. In addition to this change, a novel mRNA is formed. Analysis of cDNA clones from H378 shows that expression of this gene yields a fusion mRNA joining another gene on chromosome 1p with L-myc. Probes derived from these H378 cDNA clones show in other lung cancer lines the ubiquitous expression of a different mRNA, and in H378 possible fusion mRNA forms appearing. The sequence of this gene so far does not match any known sequence in the available data banks.

Biologic Activity of L-myc:

We have prepared a series of genomic DNA and cDNA constructs of L-myc for transfecting into primary rat embryo cells to test for biologic activity with and without mutated ras genes using the method of Land et al. (1983 & 1986). The constructs include: a 14 kb normal genomic L-myc fragment cloned into vectors with an MC29 virus LTR or with a Moloney virus LTR; a 1.8 kb tumor cell L-myc cDNA fragment encoding the entire open reading frame of the long message form placed in both orientations in the pVcos7 vector with flanking LTRs (previously used to demonstrate the biologic activity of N-myc) (Yancopoulos et al. 1985); and the 14 kb genomic fragment cloned into a retroviral vector (pDoJ-L-myc) with flanking LTRs and a neomycin selection marker (to be used in collapsing the gene into a retrovirus after transfection into packaging cells). We have begun experiments co-transfecting these vectors with mutated c-Ha-ras genes (pEJ6.6) and comparing their biologic activity with similarly co-transfected v-myc (pSV2gpt AMV LTR-v-myc) or N-myc (pRV-N-myc-1) constructs.

Preliminary characterization of the product(s) of the L-myc gene reveal a family of proteins with nuclear localization:

We have taken several approaches to identify the predicted L-myc protein products similar to the techniques used to study other myc family member proteins (Persson et al. 1984a and 1984b; Watt et al. 1985). The first used antibodies to analyze L-myc proteins in small cell lung cancer lines amplified for L-myc. The second approach employed L-myc cDNA clones for in vitro translation analysis.

We used available anti-myc anti-sera (Evan and Hancock, 1986) directed at the regions of amino acid sequence we predicted to be homologous between c, N, and L-myc and our own preparations of anti-L-myc polyclonal (rabbits) and monoclonal antibodies (mice) following immunization with synthetic L-myc peptides or an L-myc fusion protein. For in vitro translation studies a cDNA clone carrying the entire open reading frame of the 4 kb mRNA was placed into the pGEM4 vector to generate RNA in vitro.

A chimeric L-myc fusion protein was produced by incorporating another L-myc cDNA clone coding for the carboxy terminal 122 amino acids into a beta galactosidase fusion protein using the pUR vectors 291 and 292. We showed that the construct in 292 was in frame by DNA sequencing, while the 291 construct was out of frame providing a control for immunization. The fusion protein was purified on a beta galactosidase substrate affinity column (yield > 95% pure material) and used for immunization. Antisera specific for the L-myc protein were raised in rabbits that detect L-myc in immunoblots of small cell lung cancer line H510 (amplified and expressing high levels of L-myc mRNA) with enrichment in nuclear fractions, and immunoprecipitate in vitro translated L-myc proteins. In addition, in collaboration with Dr. Roger Kennett (Univ. Penn.), monoclonal anti-myc homology region antibodies detect a series of proteins in immunoblots of small cell lung cancer lines producing only L-myc mRNA and these proteins appear to be associated with the nucleus. In sum, these studies identify a series of phosphoproteins which subcellular fractionation studies show localize to the nucleus. Thus, at least some of the protein products of L-myc have similar features to those described for c-myc and N-myc (Hann and Eisenman 1984; Eisenman et al. 1985; Ramsay et al. 1984 & 1986; Slamon et al. 1986; Persson and Leder 1984).

Project #2:

Identification and Characterization of Genes Contributing to the Pathogenesis of Lung Cancer Including those in Chromosome Region 3p as well as those in Other Chromosomal Deletions Found in Lung Cancer.

Specific objectives include:

1. To determine cytogenetic changes present in lung cancer cells of various histologic types from primary and metastatic sites.

2. To determine if the deletion of chromosome region 3p(14-23) observed cytogenetically in small cell lung cancer represents a true deletion of DNA from the tumor cell using restriction length polymorphism (RFLP) probes.

3. To determine if other types of lung cancer exhibit the 3p deletion.

4. To determine if other chromosomal deletions seen in cancers such as 13q (retinoblastoma gene), 11p (Wilms' tumor gene), 1p (neuroblastoma gene), 22q11 (acoustic neurofibromas) also occur in lung cancer using appropriate RFLP as probes.

5. To find out if a chromosome #3 from a normal individual when introduced into a lung cancer cell bearing the 3p deletion can suppress malignancy.

6. To identify and clone a gene(s) which lies in the 3p region that has properties consistent with the predisposition to lung cancer and determine if lung cancer patients inherit these abnormalities.

Confirmation of the 3p Deletion in Small Cell Lung Cancer and Discovery of 3p Deletions in Non-Small Cell Lung Cancer and Mesotheliomas:

Subsequent to our report, several other investigators have confirmed the finding of a 3p deletion in small cell lung cancer (Yunis 1983, 4 of 4 cases; Falor et al 1985, 3 of 3 cases; de Leij et al. 1985, 3 of 3 cases) including primary tumor cells (Graziano et al. 1987). Others have found a 3p deletion in some small cell lung cancers and some non-small cell lung cancers (Zech et al. 1985) (see below).

Dr. Whang-Peng has continued and extended our studies. To date she has evidence of 3p deletions/ translocations in 31 of 35 small cell lung cancer tumors and cell lines. In addition, she has also found 3p deletions/translocations in 4 of 21 non-small cell lung cancers, 2 of 3 mesothelioma cell lines, 4 of 4 typical and atypical carcinoids (see site visit report by Dr. Gazdar).

Identification of Translocations Involving the 3p region of Lung Cancers:

Of great interest, recently Dr. Whang-Peng has identified several of our lung cancer lines with translocations of the 3p region including tumors with multiple 3p translocations.

Translocations of the 3p region in Lung Cancer Cell Lines (Work of Dr. J. Whang-Peng)

Cell Line	Cell Type	3p Translocations Found
H526	Small cell	t(1;3)(q12;p14)
H792	Small cell	t(3;5)(p14;p15)
H647	Adenosquamous	t(3;11)(p12;p12)
H661	Large cell	t(3;8)(p21;p23) & t(3;5)(p21;p15)
H513	Mesothelioma	t(3;4)(p21;p14)
H727	Carcinoid	t(3;20)(p14;q13) & t(3;7)(p14;q32) t(3;5)(p14;q35)

Some of these tumor lines also had a 3p deletion, while other non-small cell lines had translocations involving 3q. The translocations provide another mechanism for genetic change in the 3p region and suggest that there may actually be several sites involved, one narrowed to band 3p14, one to band 3p21, another to band 3p25, and possible 3q sites as well.

Restriction Fragment Length (RFLP) Polymorphism Analysis Shows a Deletion of DNA from 3p in Small Cell Lung Cancer:

While the cytogenetic evidence involving deletions and translocations of the 3p(14-23) region is substantial, the aneuploid nature of the tumor cells (easily hiding a translocated portion of 3p) and the presence of several copies of a cytogenetically normal chromosome #3 (which could represent chromosome #3's from both of the patient's parents) still left open the issue if the significant genetic event was a deletion or translocation. The development of RFLP's assigned to chromosome region 3p allowed us to answer this question. We have done this collaboratively, with Dr. Susan Naylor (Univ. Texas, San Antonio) beginning in 1984 and more recently with Dr. Berton Zbar (Frederick Cancer Research Center, NCI). Three RFLP probes detecting four different DNA fragments in region 3p have been used in a comparison of constitutional and tumor genotypes. DNA from tumor and normal tissue was obtained at autopsy from tumor

cell lines, resected surgical samples, peripheral blood lymphocytes, and B-lymphoblastoid cell lines.

The 3p probes used include:

1. pMS1-37 (detecting locus D3S3 on band 3p14.2 via a Msp I polymorphism) (Barker et al 1984)

2. p12-32 (detecting locus D3S2 located on band 3p21 via a Msp I polymorphism) (Gerber et al. 1986; Naylor et al 1984).

3. pH3H2 (DNF15S2) and pH3E4 derived from the lambda H3 (D1S1) probe (Harper and Saunders 1981) detecting loci on band 3p21 (Carritt et al 1986; Donlon and Magenis 1984) via Hind III polymorphisms as well as markers on chromosome #1 (D1S1).

A variety of other RFLPs on 3q as well as other chromosomes were also used.

In the two studies conducted on samples from independent patients, over 25 pairs of constitutional and tumor DNA have been informative (that is heterozygous) for at least some of the 3p markers. In all cases we have seen loss of heterozygosity in the small cell lung cancer DNA (Naylor et al. 1987; Brauch et al. 1987). The loss was not selective in that either allele of the various 3p markers could be lost. Where several markers have been heterozygous, the most conservative estimate of the data is that all have been lost (rather than a loss implicating the 3p14 or 3p21 region). In addition, a series of small cell lung cancer DNAs without matching constitutional DNA have been studied and none have shown heterozygosity for any of the 3p markers.

Two Different Mechanisms of Loss of Heterozygosity of 3p Alleles:

Examination of polymorphisms over the length of chromosome #3 (where both 3p and 3q markers were informative) suggested possible mechanisms of the chromosome 3 aberrations. In the largest group, the 3q markers remained heterozygous in the tumors while the 3p markers showed allele loss. These data are consistent with interstitial or

terminal deletion of chromosome #3 (although somatic recombination cannot be excluded). Such changes were also seen cytogenetically in the form of the interstitial or terminal deletions.

A smaller number of samples showed loss of heterozygosity at both 3p and 3q markers, findings compatible with non-disjunction/ total chromosome loss. Densitometry tracings of the tumor DNA allele compared to the amount of the same allele in the normal DNA, show examples of both hemizygous amounts of #3 DNA (equivalent to the cytogenetic finding of only one chromosome #3) or amounts of #3 DNA for the remaining allele similar to that seen in the normal tissue (equivalent to the cytogenetic findings of multiple copies of a normal appearing chromosome #3). In either event, we have been struck by the high frequency of gross deletions or total chromosome loss seen both cytogenetically and by RFLP studies.

Demonstration of 3p Allele Loss in Non-Small Cell Lung Cancer:

Dr. Zbar's group has also studied 15 informative matched pairs of tumor and constitutional DNA from non-small cell lung cancers. Loss of 3p alleles was seen in four of these including two adenocarcinomas, one bronchiolioalveolar and one epidermoid cancer (Brauch et al. 1987). We have reviewed the histology of the patients' tumors on all of these cases and confirmed they have a histologic diagnosis of non-small cell lung cancer. These RFLP studies complement the cytogenetic findings of Dr. Whang-Peng and Zech et al. (1985) of 3p changes in non-small cell lung cancer. It is of interest that work by Dr. Zbar's group, completely separate from our lung cancer collaboration, has shown loss of 3p alleles in a series of renal cell carcinomas (Zbar et al. 1987).

We conclude from these studies that there is true allele loss occurring in the vast majority of small cell lung cancer and at least 20-25% of non-small cell lung cancer.

Increased Frequency of Rare 3p Alleles for D3S3 in Small Cell Lung Cancer:

In a reference population, the 4.8 kb Msp I allele at the D3S3 locus was found only 4% (3 of 68 chromosomes tested) of the time (Barker et al.

1984). In contrast, in both Naylor's and Zbar's analysis, the allele has been detected signficantly more frequently, 23% (17/74 chromosomes) in the DNA of small cell lung cancer patients. This has also been seen by Gerber and Scoggin (1986). In non-small cell lung cancer the frequency was 15%. As stated above, neither the 4.8 or the 3.6 + 1.2 kb alleles were selectively loss from the tumor DNA. Nevertheless, the increased frequency of this uncommon allele in small cell lung cancer and possibly non-small cell lung cancer provides another genetic link to the 3p region in lung cancer.

Lack of Expression of Aminoacylase-1 in Small Cell Lung Cancer Provides Another Link to Gene Inactivation in Chromosome Region 3p:

Aminoacylase-1 is encoded by a gene assigned to region 3p21 (Naylor et al. 1982). In retinoblastoma complete loss of the 13q14 locus containing the gene for esterase D was first detected by loss of enzyme activity in tumors (Sparkes et al. 1980; Benedict et al. 1983) and only later were homozygous deletions detected using RFLPs (Dryja et al. 1986). This led into studies where a molecular probe for the 13q14 region detecting homozygous deletions allowed the identification of a candidate for the *Rb* gene (Friend et al. 1986; Lee et al. 1987). This complete absence provided an important signpost in identifying the *Rb* gene candidate.

In attempting to retrace these steps in the 3p region Dr. York Miller (VA Medical Center, Univ. of Colorado) in collaboration with Dr. A. Gazdar of our group has studied the expression of aminoacylase-1 in 29 of our small cell lung cancer lines (Miller et al. 1987). Both enzyme activity assays and immunologic detection of the protein were employed. Six of 29 small cell lung cancer samples failed to express detectable aminoacylase-1 activity by enzyme assay, with low levels of enzyme activity seen in 13 additional small cell samples, whereas only one of 34 other tumor lines including many of our non-small cell lung cancer lines had a low level of expression. The immunoassay showed lack of enzyme antigen in the enzyme activity negative lines confirming absence of expression. These results suggest that genes on the cytogenetically normal appearing chromosome #3 are frequently inactivated or supressed.

Also these results suggest aminoacylase-1 may be a marker closely linked to the small cell lung cancer locus.

c-Raf-1 Proto-oncogene located on 3p25 Region is Expressed in Small Cell Lung Cancer:

The gene for the c-raf-1 proto-oncogene has been assigned to chromosome region 3p25 distal to the small cell lung cancer 3p(14-23) locus. Because of this chromosomal location and the frequent finding of terminal deletion of the 3p region several years ago we began a collaboration with Dr. Ulf Rapp (NCI, FRCR) to look at the structure and expression of c-raf-1 in small cell lung cancer. Work by Drs. I. Kirsch and G. Hollis showed no evidence for c-raf-1 gene abnormalities using the available probes. However, nearly all of the small cell lung cancer lines express c-raf-1 mRNA and protein (Rapp et al. in preparation). In order to determine if this expression is activated in the lung cancer lines, the status of of c-raf-1 expression in normal lung needs to be studied.

Other Chromosome and DNA Changes Found in Lung Cancer:

Deletions of 13q are associated with retinoblastoma (Cavanee et al. 1983 and 11p deletions have been seen with Wilms' tumors (Koufos et al. 1984). In our work with Dr. Naylor, 4 of 9 small cell lung cancer samples had evidence of loss of chromosome 13 alleles. Dr. Whang-Peng found cytogenetic evidence for 11p deletion in 2 of 16 small cell lung cancers (1982a) From all of these studies we conclude that genes on several different chromosomes are likely to be involved in the pathogenesis of lung cancer.

Project #3

Growth Factors (including Autocrine Growth Factors) in the Pathogenesis of Lung Cancer

Lead Investigator: Frank Cuttitta, PhD
Objectives

Overall Objective:

To identify autocrine and external growth factors involved in the pathogenesis and growth of lung cancer and develop methods for exploiting this information in the prevention and treatment of lung cancer.

Specific Objectives:

1. To determine the specific growth factor requirements of lung cancer cells in vitro by adding purified factors to tumor cells growing in defined media and determine which stimulate or inhibit growth.

2. To establish tumor cell lines that are able to replicate in media with as few growth factors as possible to determine the extent to which lung cancer cells can grow independently of exogenously added factors.

3. To identify the production of specific peptide hormones and growth factors by lung cancer cells, particularly those growing in serum free conditioned media.

4. To demonstrate that the factors produced by lung cancer cells when added to lung cancer cell cultures in purified form stimulate or inhibit tumor cell growth in vitro.

5. To demonstrate that specific receptors exist on lung cancer cells for the growth factors produced by the same cells.

6. To demonstrate that specific antibodies or antagonists directed against the peptides produced by lung cancer cells (or against the receptors for these peptides) will inhibit the growth of the tumor cells in vitro and in vivo in xenografts.

7. To determine if the anti-GRP antibodies will inhibit normal fetal growth and inhibit the early stages of carcinogen induced neuroendocrine proliferation in experimental animal systems.

8. To relate the production of autocrine growth factors, especially GRP, to growth stimulation of preneoplastic bronchial epithelial lesions

and lung cancer cells via expression of cellular proto-oncogenes and place these events temporally in relationship to the development of 3p chromosomal deletion/translocations.

9. To test these findings in clinical trials.

Non-small cell lung cancers require a different hormone supplementation for serum -free medium replication:

In more recent studies we have applied the same methodology to work out a serum free, growth factor supplemented media for non-small cell lung cancer (Brower et al. 1986; Gazdar and Oie, 1986; Minna et al. 1986; and the annual report of Dr. Gazdar). These studies showed that non-small cell lung cancers had different growth factor requirements than small cell lung cancers. These requirements included: 1.) the need for attachment factors such as collagen; 2.) other peptide hormones such as EGF and T3; 3.) other constituents such ethanolamine, phosphoryl ethanolamine for adenocarcinoma, and cholera toxin for squamous cell cancers (Minna et al. 1986).

GRP as an autocrine growth factor for small cell lung cancer:

The key to dissecting the role of bombesin in small cell lung cancer growth proved to be a mouse monoclonal antibody (2A11) which we produced and were able to show using synthetic peptides had precisely the same binding specificity for bombesin congeners as the bombesin receptor (Cuttitta et al. 1985a; 1985b). This antibody blocked the binding of labeled bombesin to its receptors on small cell lung cancer and rat brain membrane preparations, and blocked the effect of exogenously added bombesin to stimulate the clonal growth of small cell lung cancer. Most importantly, the antibody was able to inhibit the growth of small cell lung cancer in defined medium (without added bombesin), and to inhibit the growth of small cell lung cancer xenografts in athymic nude mice (Cuttitta et al. 1985a; 1985b).

Recently, this work has been confirmed by another group who made another monoclonal antibody with similar specificity for GRP/bombesin that also inhibits the growth of small cell lung cancer in vitro and in

xenografts (Dienhart et al. 1987). Taken together the evidence demonstrates that GRP can function as an autocrine growth factor for at least some small cell lung cancers and prompted our current clinical trial of the 2A11 anti-GRP monoclonal antibody in patients with lung cancer (see the details of this trial in the annual report by Dr. Mulshine).

Expression of multiple forms of GRP gene associated peptides in small cell lung cancer and human fetal lung:

Work by Dr. James Battey and Edward Sausville (see Dr. Battey's annual report) had indicated the presence of three distinct preproGRP mRNAs generated by alternative splicing in small cell lung cancer (Sausville et al. 1986). These mRNAs contained in addition to a signal peptide, GRP 1-27, and open reading frame coding for several versions of predicted GRP gene associated peptides. Because of the important role of GRP in autocrine growth of small cell lung cancer we wanted to demonstrate the existence of the predicted peptides. Thus, we generated antisera against four synthetic peptides predicted from the cDNA sequence data including peptides present in GRP, the constant region of the GRP gene associated peptide, and two of the three C-terminal variable regions (forms I and III) of the associated peptide.

We have shown these four anti-sera to be high titered and very specific in their ability to distinguish the different peptides. They were then used in immunoblots of small cell lung cancer lines and readily detected preproGRP (Cuttitta et al. 1987). This demonstrated that not only GRP but the various GGAP peptides were translated in small cell lung cancer. In addition, the antibodies could readily detect the various GRP and GGAP peptides in histologic sections of small cell lung cancer and human fetal lung demonstrating the presence of the alternate forms during normal development. In fact, serial sections of human fetal lung revealed that all of the various GRP and GGAP peptides were produced in the same neuroendocrine cells and neuroepithelial bodies (Cuttitta et al. 1987). Dr. Battey has done extensive other studies on the molecular genetics of preproGRP and the expression and processing of these peptides in various experimental systems including Swiss 3T3 cells transfected with preproGRP (see Dr. Battey's annual report).

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Small cell lung cancer growth is inhibited by the tachykinin peptide physalaemin:

In looking for other neuopeptides that could regulate the growth of small cell lung cancer we became aware of reports that physalaemin, a member of the tachykinin family, could stimulate the growth of some mesenchymal cells. However, we found that physalaemin at picomolar concentrations inhibited the growth in vitro of four different small cell lung cancer lines exposed to the peptide in serum free medium (Bepler et al. 1987).

Lung cancer cell lines as well as other tumor cell lines can be adapted to grow in medium with no added growth factors:

With the discovery of GRP as an autocrine growth factor we were interested in whether other such factors were made. This time, rather than adding growth factors to serum free medium, we attempted to remove all factors from the medium and asked whether or not the lung cancer cells could produce all the factors (or activate all the transducing signals) required for their growth. We transferred the tumor cells first to HITES medium described above, and then after varying periods of time (1-4 weeks depending on the individual cell line), into basal RPMI-1640 medium without any supplementation (R0 medium). The tumor cells continued to replicate in this R0 medium although their growth was dependent on maintaining a high cell density (Cuttitta et al. 1987). Currently we have

successfully adapted and cultivated 19 tumor cells lines in R0 for periods of 4-28 months. These include lung cancer lines of several histologic types, colon cancers, and a pancreatic cancer line.

Preliminary characterization of the conditioned medium of tumor cells adapted to grow without added factors:

Conditioned R0 medium from these lines would stimulate the growth of serum dependent lines acutely transferred to serum free medium suggesting the secretion of growth factors into the R0 medium. We selected the conditioned medium of two small cell lung cancer lines (H345 and H510) for further study. Both of these lines, maintained for long periods of time in R0, express L-myc and a number of neuroendocrine

markers including GRP, and both respond to exogenously added GRP by elevating intracellular Ca2+ levels (see annual report of Dr. Sausville).

Ultimately, any peptides produced into the culture fluid have to be chemically characterized for final identification. As a test of this, in collaborative studies with the groups of Dr. John Walsh at ULCA (including Drs. Joseph R. Reeve, and Steven R. Vigna) and Dr. Jack Shively (City of Hope) the conditioned medium was fractionated on HPLC and a combination of radio-immunoassays and sequencing of isolated peptides confirmed the production of GRP and the GRP gene associated peptide (GGAP), into the conditioned medium.

Serum free conditioned medium from small cell lung cancer lines adapted to grow in R0 elicits the production of antibodies against many different peptide hormones:

Rather than establish a large number of immunoassays for many peptide hormones we developed a new way to screen for the presence of peptides into the conditioned medium, namely the ability of the R0 conditioned medium to elicit antibody production against specific hor-

mones. The conditioned medium was adsorbed to talc or a C18 Sep Pak, eluted with acetonitrile, lyophilized to powder, redissolved, conjugated to KLH and used to immunize rabbits to produce high titered anti-conditioned medium antisera. These antisera were tested in plate binding assays using a variety of purified peptide hormones as targets. In this manner we found that antibodies against specific peptides could be induced by the conditioned medium free of serum or exogenously added hormones.

The highest titers of antibodies were elicited against transferrin and insulin, or insulin like growth factors as well as GRP and calcitonin (the latter two being peptides known to be produced by small cell lung cancer). Glucagon like peptides were identified which potentially could inhibit the action of the insulin-like factors. In addition, vasopressin, MSH, and enkephalins were detected which small cell lung cancer can produce, while ACTH (a portion of the pro-opiomelanocortin molecule) was not picked up in this assay. It is interesting to note that with Dr. Moody we had previously demonstrated the production and secretion of neurotensin by

our small cell lines (Moody et al. 1985) but we have not been able to demonstrate any effect of this peptide hormone on the growth of the lung cancer cells, nor its production into R0 conditioned media by the antibody induction assay. Since insulin and transferrin were the two most potent components of the HITES medium (Simms et al. 1980) as well as being general requirements in other serum free media for the growth of cultured cells (Bottenstein et al. 1979) we focused on these two peptides.

IGF-I stimulates the growth of lung cancer cells in vitro:

Prompted by finding insulin and IGF-1 immunoreactivity in the conditioned media we compared recombinant IGF-1 (AmGem) with insulin in serum free growth assays of small cell lung cancer. These showed that IGF-1 was 10-100 times more potent than insulin in stimulating growth. In addition, a monoclonal antibody (anti-IR3) able to distinguish the IGF-1 from the insulin receptor and a powerful antagonist of IGF-1 receptor-mediated stimulation of DNA synthesis (Kull et al. 1983) completely inhibited both insulin and IGF-1 induced proliferation of small cell lung cancer (Nakanishi, et al 1987). This is consistent with the report that

insulin mediates part of its mitogenic effct on human skin fibroblasts through the IGF-1 receptor (Van Wyk et al. 1985). Thus, we found no evidence that insulin could stimulate small cell lung cancer growth through its own receptor as described for some human fibroblast lines (Flier et al. 1986), and points to IGF-1 like molecules as the biologically active ones in small cell lung cancer.

Immunoblot detection of IGF-1 like peptides in the conditioned serum free media of lung cancer cells:

Recently cDNA analysis has revealed several forms of IGF-1 mRNAs produced by alternative splicing in human liver (Rotwein 1986) including a short (IGF-1A) and a long (IGF-1B) form (Figure 2). These mRNAs predict the existence of various IGF-1 associated peptides in the C-terminal region including sequences which could generate biologically active, amidated peptides. We have had synthetic peptides predicted by the cDNA sequences prepared (Applied Biosystems Inc. (Foster City, CA) and used these to generate high titered anti-IGF-1 anti-peptide sera. The anti-serum directed against the IGF-1 synthetic peptide readily detects IGF-1 like molecules in conditioned R0 media from small cell lung cancer lines

N417 and H345 using immunoblot assays. It is of interest to note that H345 produces GRP and expresses L-myc but not c-myc, while N417 does not express GRP and is amplified for and expresses abundant quantities of c-myc suggesting that IGF-1 like molecules, in contrast to GRP, can be produced by either the classic or variant tumor cell type.

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Monoclonal Antibody Work

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06578-04 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

The Molecular Biology of the Mammalian GRP Gene

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

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Others:	Anne-Marie Lebacq-Verheyden, M.D., Ph.D.	Guest Researcher	NCI-NMOB
	Sanford Markowitz, M.D., Ph.D.	Medical Staff Fellow	NCI-NMOB
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SECTION

Genetics, Molecular Biology and Immunology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	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 structure, function, and expression of mammalian prepro gastrin releasing peptide (GRP) genes was studied. Experiments were performed to a) characterize the structure and regulation of the rat prepro GRP gene, b) overexpress the human GRP prohormone protein to analyze post-translational processing and peptide hormone function, c) test the autocrine growth hypothesis by introducing a constitutively expressed GRP gene into cells which respond to exogeneous GRP hormone by increased cell growth.

A. Rat prepro GRP gene. The structure and entire nucleotide sequence of the rat prepro GRP gene was determined, using genomic clones and cDNA clones isolated from a rat brain cDNA library. In contrast to the human gene, there is no alternative RNA processing observed in rat tissues expressing this gene. The coding regions for GRP and its associated peptide are evolutionarily conserved, consistent with a biologic function for both domains. The rat GRP gene is transcribed from a tissue specific promoter in the brain, creating an alternative route for regulating gene expression.

B. The human prepro GRP gene was successfully overexpressed in a novel baculovirus protein expression system. Using this model system, sufficient protein can be produced to allow analysis of post-translational processing events needed to generate biologically active peptides, and purify those peptides to establish function.

C. A constitutively expressed human GRP gene was introduced into mouse embryonic Swiss 3T3 fibroblasts using DNA transfection. These fibroblasts, which respond to added GRP by cell division, failed to fully process the GRP prohormone into biologically active peptides and consequently showed no growth changes. This experiment establishes that processing of GRP prohormone is restricted to specific cell types.

GPO 914-916

PROJECT DESCRIPTION

The Molecular Biology of the Mammalian GRP Gene

Professional Staff:

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	James Way	Microbiologist	NCI-NMOB

Introduction

Gastrin releasing peptide is a 27 amino acid neuropeptide representing the mammalian homologue to the amphibian mitogenic peptide bombesin (1). These two peptides share an identical carboxy-terminal, -amidated heptapeptide, which is necessary and sufficient for binding to high-affinity cell surface receptors, initiating the physiologic responses observed in central and peripheral neurons, and in secretory cells (2). In addition, bombesin and GRP are potent mitogens for a number of cultured cells, including Swiss 3T3 mouse embryo fibroblasts (3), human bronchial epithelial cells (4), and small cell lung cancer cells (5,6). The latter cells both synthesize and secrete a biologically active bombesin like immunoreactive peptide (7,8,9), shown by molecular analysis to be the gene product of the prepro GRP gene (10). When a monoclonal antibody whose epitope overlaps the GRP carboxyl heptapeptide and prevents ligand binding to surface receptors, the growth of some SCLC cell lines is impaired both in a soft agar cloning assay and in nude mouse xenografts (6). These data suggest that under some circumstances GRP could stimulate the growth of the same cells which secrete it. Such an autocrine growth stimulation is thought to contribute to the acquisition of a malignant phenotype (11). In support of this hypothesis, both autocrine growth stimulation and tumorigenicity were induced in a factor dependent hematopoietic cell line given a highly expressed GM CSF growth factor gene (12). Further, rat fibroblasts expressing cell surface receptors for TGF- showed more vigorous clonal growth in soft agar and increased tumorigenicity in nude mice after transfection with a constitutively expressed TGF- gene. This malignant progression was reversible when antibodies to TGF- were added to medium surrounding the cells (13). Our laboratory is currently undertaking a molecular analysis of the mammalian prepro GRP gene, in an effort to both define its structure, regulation and expression, as well as to explore the biologic properties of this gene in both normal and malignant cells.

Progress Report

Cloning and Structure of Human prepro GRP gene

A collection of human prepro GRP cDNA clones were obtained by screening several SCLC cDNA libraries with a human GRP probe (10). Analysis of these clones revealed a predicted prepro GRP translation product consisting of a signal

sequence, the 27-amino acid GRP neuropeptide and a novel GRP-associated extension peptide representing the carboxyl portion of the translation reading frame. Three forms of cDNAs were found in the structural characterization of these clones, which differ only in the region encoding the GRP-associated peptide (G-GAP). Each of the three types (I, II, III) would direct the synthesis of a distinct G-GAP, as well as identical GRP peptides (10). Comparison of the sequence of the three classes of cDNA clones with a genomic clone derived from a human placental library shows that all three forms of transcripts are synthesized from the primary transcript of a single three exon gene. This primary transcript is spliced in the same fashion in all three types to remove the first intervening sequence, which is positioned at the carboxyl end of the GRP peptide. In contrast, alternative RNA processing at two splice donor and two splice acceptor sites bordering the second intervening sequence generates the three forms of the G-GAP peptides. Thus, at least four peptide hormones could be generated from a single human GRP gene by a combination of alternative RNA processing and post translational processing (10).

Using the derived cDNA clones, specific probes for GRP mRNA expression and specific antisera to defined regions of the predicted translation product were generated (14). Studies using the probes have established a number of interesting features about prepro GRP gene expression. Detectable steady-state levels of GRP mRNA are found in most cultured SCLC cells, and in no non-SCLC cells or lymphoid cells examined to date. The correlation between levels of GRP mRNA and bombesin like immunoreactivity is very good, implicating human GRP as the peptide responsible for the observed immunoreactivity in SCLC. S_1 nuclease and immunohistochemical studies performed on samples from both human fetal lung and SCLC clearly demonstrate the existence of prepro GRP mRNA, GRP, and the various predicted forms of G-GAP peptides in both normal and malignant tissues (14). Embryonic human lung (20-22 week gestation) showed high levels of GRP gene expression, consistent with the hypothesis that GRP might act as a modulator of growth and development in mammalian lung.

Karyotypic changes in tumors have frequently been correlated with altered expression of genes encoding growth factors, growth factor receptors, and protooncogenes. These DNA rearrangements can alter the structure or regulation of these adjacent genes, contributing to the deregulated growth properties of tumor cells (15). In collaboration with Drs. Ilan Kirsch, Greg Hollis, and Dr. Wes McBride, we mapped the human prepro GRP gene to chromosome band 18q21 using both human-rodent somatic cell hybrids and *in situ* hybridization to metaphase chromosomes (16). No currently identified karotypic abnormality in human SCLC involves 18q21; nevertheless, karyotypic abnormalities in tumors and inherited disease states which involve 18q21 may now be examined for correlated changes in the structure and expression of the GRP gene.

Regulation of Expression

Significant differences in the steady-state levels of pre-pro GRP mRNA were observed in a series of cultured cell lines analyzed by RNA blots and S_1 protection. These cells provide an opportunity to study the mechanisms responsible for regulating GRP gene expression, and peptide hormone gene expression in general. Several mechanisms known to be important in the regulation of eukaryotic genes

may be important in determining the steady-state level of GRP mRNA. Changes in the level of RNA polymerase II-directed primary transcription can clearly affect the resultant steady-state amounts of mature mRNAs. Transcription regulation by this mechanism was clearly shown to be a primary determinant in beta globin switching of maturing chicken embryos where the level of primary transcription was measured by ^{32}P-labelling of nuclear run-on transcripts (18). Changes in the post-transcriptional stabilities of mature mRNA may also modulate steady-state levels, as was demonstrated for c-myc mRNAs on a series of mouse B-lymphoid cell lines. In these studies, the level of primary transcription from the c-myc gene is equivalent, and the eight-fold difference in steady-state mRNA levels observed was explained by a greatly increased half-life of c-myc mRNA in the murine plasmacytoma cell lines containing abundant message (18). Transcriptional activation of genes has been associated with chromatin structural changes, including increased sensitivity to DNase I (19) and hypomethylation of CpG dinucleotides (20). Methylation status and DNase I sensitivity of Ig genes are altered in B-lymphocytes that undergo the gene rearrangements preceding gene expression (21), and are also coincident with changes in chicken (22) and human (23) globin gene expression. Finally, cis-acting transcriptional enhancer elements have been described which dramatically alter the transcriptional activity of genes in both a tissue-specific and non-specific fashion (24).

Our laboratory has initiated a series of experiments to elucidate which of the molecular mechanisms govern the specific expression of prepro GRP in mammalian cells. Analysis of the genomic DNA flanking the gene showed no obvious differences between the restriction maps of cultured cells expressing mRNA and their non-expressing counterparts. These data indicate that no somatic structural DNA change detectable with these methods is correlated with altered expression. Preliminary studies assessing the chromatin structure of the prepro GRP gene show no changes in methylation correlating with changes in expression. DNase I sensitivity is currently being analyzed, which may reveal changes in sensitive sites correlating with GRP gene expression. To identify cis-acting DNA regulatory elements, we have constructed hybrid genes fusing portions of the human prepro GRP locus with the chloramphenicol acetyl transferase (CAT) gene. The effects of various genomic GRP DNA segments on transcription can then be easily monitored by transfecting those hybrid genes into various cell lines, and determining the level of CAT enzyme that is transiently expressed (25). We are currently using these constructs to evaluate the GRP promoter strength in a variety of cultured cells, and also to identify cis-acting transcription regulatory regions in the prepro GRP locus. We plan to continue these ongoing studies, as well as exploring other potential regulatory mechanisms in the future.

Normal Properties of prepro GRP Gene in Non-malignant Cells

Peptides synthesized from the mammalian GRP gene are important transducers of cell-cell interactions in a variety of tissues. To achieve a more complete understanding of the biologic repertoire of the prepro GRP gene, it is essential to establish a mammalian animal model system for experimentation. Observations made in this context should have predictive value in highlighting GRP functions in humans. In addition, molecular comparison of homologous genes in two different mammalian species often reveals significant structural and regulatory motifs by virtue of their evolutionary conservation. The Sprague-Dawley rat is used by

many neurobiologists for experiments mapping the function and distribution of neuropeptides, making it an appropriate animal model for this study. A first step in this comparative analysis involves cloning and characterizing cDNA and genomic clones from rat. We have recently completed the initial phases of this work. Structural studies show that the rat prepro GRP gene is highly conserved over its entire three-exon structure, including the regions encoding the G-GAP. Complementary DNA clones derived from a rat brain cDNA library suggest that a longer form (1.5 Kb) of the GRP mRNA is transcribed uniquely in brain, and that this novel species initiates transcription from a promoter 5' to the promoter identified in human SCLC or GRP mRNA derived from rat duodenum. S_1 nuclease and RNA blot analysis confirms these results. We are currently exploring in detail the structural and functional properties of this new promoter, and hope to define which brain cells are involved in synthesizing the longer transcript. The existence of an additional promoter activated in a tissue-specific fashion raises many interesting possibilities for regulatory mechanisms, which will be addressed in future experiments.

One of the novel features of the human prepro GRP gene is the alternative RNA processing mechanism which generates three distinct G-GAP peptides. S_1 nuclease studies analyzing the homologous region of the rat gene show that no such alternative splicing is occurring in the rat. In contrast to the human, a single G-GAP peptide sequence is predicted from the rat GRP mRNA population in both the gut and the brain. The single G-GAP peptide would show strong amino acid sequence homology with human G-GAP from III, providing circumstantial evidence that this form may be biologically relevant.

Expression of GRP Prohormone in Recombinant Baculovirus

To determine the functional importance of G-GAP, it will be essential to isolate purified preparations of each G-GAP peptide form. No abundant natural source for these peptides is known. Moreover, all human cell lines examined that synthesize GRP make all three G-GAPS, confounding form-specific purification. We are utilizing a baculovirus protein expression system to solve this problem. Briefly, a recombinant baculovirus vector is used to exchange the prepro GRP coding sequences for non-essential late viral protein (polyhedrin) coding sequences. Thus, prepro GRP is expressed in high quantities at 48-72 hours post-infection of the moth ovum (SF9) host cell. In the insect cell host, many post translational protein modifications including glycosylation occur maximizing the opportunity to synthesize a biologically active product. We have used this same baculovirus protein expression system to synthesize and purify the hemagglutinin-neuraminidase protein from human type 3 parainfluenza virus from cloned cDNA. This protein was appropriately glycosylated, transported to the cell surface, and was biologically active. All epitopes previously mapped to the active sites in this molecule were conformationally unaltered on the recombinant protein, suggesting that this represents authentic HN glycoprotein (Coelingh et al., manuscript in preparation). This system has been used by others to produce biologically active IL-2 from a cDNA prohormone coding region after appropriate removal of signal peptide (26). Our initial attempts using this system to produce GRP-GGAP I prohormone are promising. The cells clearly synthesize and secrete bonafide prohormone after accurate removal of the signal sequence by signal peptidases (27). The prohormone is subsequently cleaved at or near a number of specific basic amino acid residues in the G-GAP molecule. Whether or

not these cleavage events are part of normal prohormone processing, or the result of random proteolytic digestion, awaits the elucidation of the normal processing pathway in cells which normally express GRP prohormone. This normal processing pathway is currently under investigation. We plan to use this technique to isolate all relevant forms of G-GAP peptides for functional characterization, assessment of biologic activity and identification of any potential G-GAP peptide receptors in tissues where the gene is expressed.

Investigation of Autocrine Growth Regulation

The autocrine growth hypothesis predicts that growth deregulation of cells may occur if genetic events resulted in constitutive expression of a growth factor gene. This growth factor would be secreted, bind to cell surface receptors, and transduce a continuous proliferative stimulus. As described earlier, GRP-mediated autocrine growth stimulation has been implicated in the growth deregulation of some human SCLC cells (6). To test the generality of this model, we attempted to reconstitute an autocrine growth stimulation system by giving murine embryo Swiss 3T3 fibroblasts a deregulated prepro GRP gene. These cells possess a well characterized mitogenic response to GRP peptide after serum starvation (3), mediated by high-affinity cell surface receptors (28), but do not normally transcribe their endogenous prepro GRP gene.

A panel of transfectants was established, expressing varying amounts of prepro GRP mRNA. A comparable series of clonal transfectants was generated, expressing the GRP coding sequences in an antisense configuration to serve as a control. No growth advantage was observed in "sense" lines, when compared to a comparable "antisense" example. Both immunoprecipitation and Western blot analysis demonstrate that these cells secrete GRP prohormone with the signal sequence released; no further post translational processing had taken place (29). To generate a GRP peptide capable of binding at nanomolar concentrations to high affinity receptors, the prohormone must be processed in at least three steps (1) trypsin-like cleavage of the 15 Kd proGRP at a dibasic Lys-Lys in the sequence Met-Gly-Lys-Lys-Ser, where Met is the carboxyl-terminal amino acid of 3 Kd GRP_{1-27}, (b) the removal of the two basic residues, and (c) the cleavage of glycine which serves as the amide donor for the subsequent -amidation of the methionine. No evidence of these processing events was observed in the Swiss 3T3 transfectants, which may explain the failure to generate autocrine growth stimulation. However, it should be noted that to achieve autocrine growth stimulation, biologically active GRP must bind to receptors, and then transduce a mitogenic signal through second messengers that include phosphoinositol turnover and calcium flux (30). Failure at any of these steps could in theory also account for the observed lack of autocrine growth stimulation. From these experiments, it is clear that GRP may function as an autocrine growth factor only in certain very specific cell types which possess the necessary enzymatic activities essential for processing biologically active GRP from its inactive prohormone precursor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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PROJECT NUMBER

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October 1, 1986 to September 30, 1987

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

Chromosomal Abnormalities that Highlight Regions of Differentiated Activity

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

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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.)*

Association of a specific chromosomal abnormality with a specific tumor type is well established and may reflect mechanisms of oncogenesis peculiar to that tumor. Alternatively, it may be that these associations reflect that particular differentiated state of the malignant cell, consistent with the model that rearrangements occur only within chromatin in an "active" configuration. Using this concept as a predictive and testable hypothesis, we are investigating the relationship of specific chromosomal abnormalities to certain tumors. Our focus at present is on diseases of the hematopoietic system. This research program requires expertise in a number of distinct biological techniques. We have established this technical expertise which includes 1) our capability to grow and maintain a wide array of primary cells and cell lines, 2) our facility is doing basic cytogenetic analyses, as well as the more involved procedure of chromosome in situ hybridization, and 3) our constantly updated ability to utilize the very newest of molecular biological techniques to clone, map, sequence, and perform expression studies on DNA segments of interest.

We previously demonstrated that in a T cell and B cell tumor with a particular chromosomal abnormality (inv 14) we could account for the occurrence of the inversion by a site-specific recombination event between the Ig and TCR loci despite the fact that these loci are felt to be distinct and disparately activated. One measure of "accessibility" of genomic DNA is to analyze transcriptional activity derived from it. Perhaps, a naive view, but one that we will propose for the sake of this argument, is that if DNA is accessible to a RNA polymerase it might very well be accessible to a recombinase. We, therefore, surveyed T cell tumors and normal polyclonal T-cell populations for evidence of immunoglobulin heavy chain variable region transcription. Contrary to models of distinct T cell/B cell lineage divergence, we found evidence for immunoglobulin heavy chain variable regions transcription in T cells. This finding speaks to important issues in lymphocyte development and may have relevance to the occurrence of cancer in these cells as well.

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PROJECT DESCRIPTION

Chromosomal Abnormalities that Highlight Regions of Differentiated Activity

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Objectives

Long Term

1. To define the necessary and/or sufficient features for chromosomal breakage and rejoining in different cell types.

2. To determine if chromosomal breakage disorders represent an exaggeration of normal events or a novel pathology.

3. To use the occurrence of cell-type specific chromosomal aberrations as an in-road to the exploration of differential gene activation during development.

4. To contribute to the understanding of how gene rearrangements mediated by chromosomal aberrations alter the regulation of the affected loci.

Short Term

To study chromosomal aberrations in hematopoietic cells, particularly:

1. To determine the frequency and cell type distribution of inversions and translocations of human chromosome 14 in normal, "premalignant", and malignant conditions and explore whether there is evidence for selective or random associations between particular breakpoints and particular transformed or proliferative states.

2. To clone and sequence the breakpoints from "normal" and malignant cells carrying inversions or translocations of chromosome 14. To assess from sequence data, if possible, how the chromosomal aberration occurred and what loci were involved.

3. To study the genomic activity of loci involved in translocations and inversions of chromosome 14 in corresponding cells in which the chromosomal aberration has or has not occurred, and thereby determine if the aberration has caused deregulation or altered expression of these loci.

4. To continue our studies on the mechanisms of oncogene deregulation as a result of chromosomal rearrangements in lymphoid malignancies.

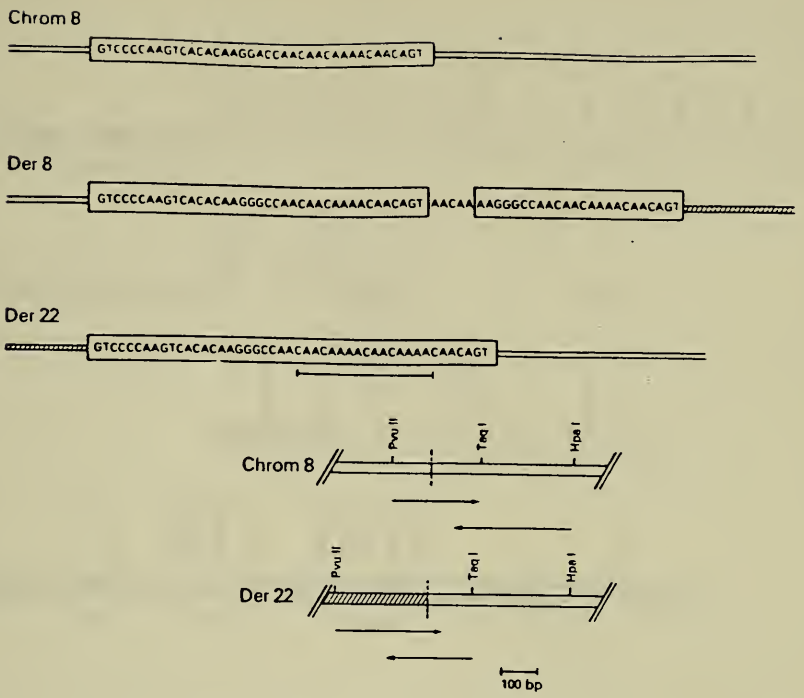
Major Findings

Analysis of a Variant Translocation in Burkitt's Lymphoma

It is compelling that the characteristic translocations associated with Burkitt's lymphomas invariably involve as one of the partners in the reciprocal exchange one of the immunoglobulin gene loci, heavy chain (14q32), kappa (2p11), or lambda (22q11). Thus, the chromosomal aberrations of a B cell tumor consistently involve the chromosomal regions in which reside the genes that encode the particular differentiated function of the cell. As mentioned previously in the most common t(8;14) translocations the analyses of breakpoints suggested the involvement of the VDJ and/or switch recombination enzyme systems. We were interested in comparing the breakpoint seen in a variant translocation [t(2;8) or t(8;22)] with the more common t(8;14) to see if a common mechanistic thread could be discerned. G. Lenoir had reported a correlation between the light chain produced in tumor cells carrying variant translocations and the particular chromosomes involved; kappa producing tumors were correlated with translocations of the kappa encoding chromosome 2 [t(2;8)], lambda producing tumors were correlated with translocations of the lambda encoding chromosome 22 [t(8;22)] (1). This was despite the fact that it was the normal untranslocated chromosome whose kappa or lambda gene was transcribed. Thus it appeared that both chromosomes 2 or 22 were accessible at a point in time, one allele underwent functional activation, the other translocation. In our interest in uncovering common themes of translocation we therefore chose to analyze not only a variant translocation but an exception to the Lenoir correlation, a Burkitt's cell with a t(8;22) that expressed kappa light chain. Salient features of this analysis (2) included the findings that there were no obvious recombination targetting signal sequences found at the breakpoint junction of the native chromosome 8, nor on a related segment of chromosome 22, nor the derivative chromosomes 8 or 22. Analysis of the breakpoint therefore did not suggest nor necessitate involvement of the VJ or switch recombination systems in the translocation. The translocation was associated with a deletion of about 32 base pairs of DNA from chromosomes 22 and the rough duplication of about 39 base pairs from chromosome 8 at the t(8;22) junction (Figure 1). The now "classic" features (3) of shift of promoter utilization in c-myc from P2 to P1 and targeted mutation of the 1st non-coding c-myc exon were dramatically demonstrated here. Only the translocated c-myc gene was transcribed. An interesting theme did begin to emerge, however, from this analysis. The violation of the Lenoir correlation was more apparent than real. Activation of the lambda locus was indeed found in this tumor. The c-myc proto-oncogene had broken 3' of its coding sequences and joined 5' of a conventional VJ rearranged lambda C3 gene (Figure 2). This event not only argued for the genomic activation of the lambda locus in this cell but also contrasted with the general finding that lambda gene rearrangements are not found in kappa producing cells (4).

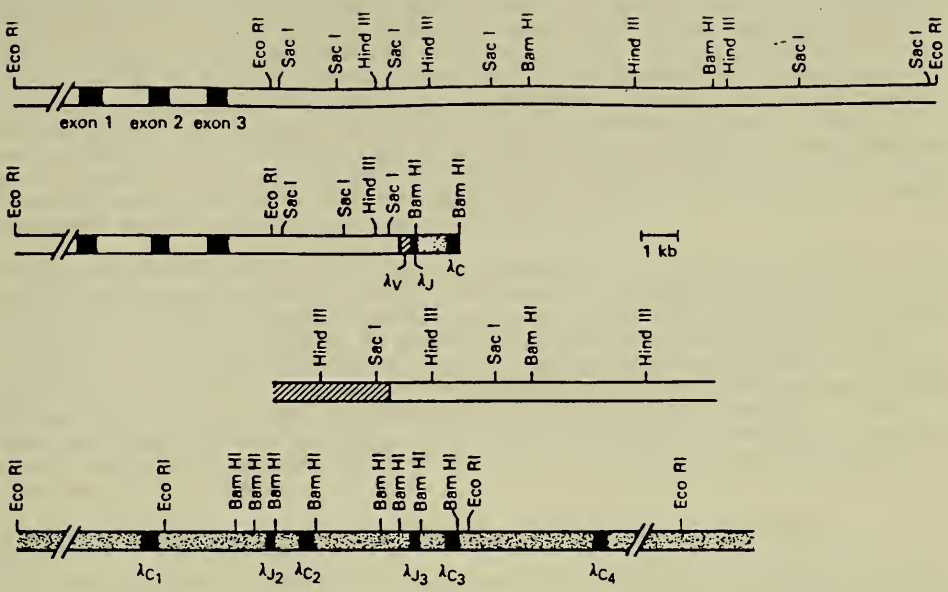
Translocations that Highlight Chromosomal Regions of Differentiated Activity

So, in Burkitt's lymphoma chromosomal aberrations were occurring to regions active in the differentiated function of B cells. Would chromosomal aberrations



involve regions of differentiated activity in non-lymphoid cells? DNA sequence at the t(8;22) junction. There is a rough duplication on a 39 bp sequence from chromosome 8 followed by a second incomplete copy on the derivative 8 chromosome. No such sequence was found on a related stretch of the germline chromosome 22 from which about 32 bps were deleted.

Clearly globin gene expression is a prototype of a cellular differentiated function. We, therefore, investigated chromosomal abnormalities in malignancies of erythrocyte precursors. We were able to analyze the karyotypes of two patients with erythroleukemia and found numerous chromosomal abnormalities among which, however, were breaks within the chromosomal bands encoding the alpha and beta globin gene clusters. The cell line K562 provided a further instructive example. It was derived by Lozzio and Lozzio from a patient with CML (5) but soon was noted to be inducible for globin synthesis and therefore was studied in numerous laboratories interested in globin gene regulation. At some point it was noted to have become less inducible and more low-level constitutive for globin synthesis. We cytogenetically analyzed early (inducible) and late (constitutive) passages of this line and found them remarkably similar in chromosome number and morphology except, strikingly, the later passage carried an additional translocation between chromosomes 11 and 13, the breakpoint on 11 being within the region to which (6) had previously localized the beta globin gene cluster.



Genomic activity of the Ig lambda locus in a t(8;22) translocation exemplified by VJ recombination activity. Top line - germline c-myc locus. 2nd line - der 8 chromosome. 3rd line - der 22. 4th line - germline chromosome 22 Ig lambda constant region locus.

We have analyzed both passages of this cell line by conventional Southern blot analysis using beta, pseudo beta, gamma, and epsilon globin probes as well as insulin and Ha-ras (also found in the same chromosomal band) without finding evidence of a difference between the two. Early attempts at pulsed field gradient gel analysis do show some differences between the two passages, but it is unclear whether these reflect the translocation or differences in methylation patterns to which the enzymes we use are sensitive.

If chromosomal aberrations are indeed highlighting regions of genomic activity in differentiated cells then one might suspect that chromosomal aberrations might often be cell-type specific. To test this possibility we did a karyotypic analysis of phytohemagglutinin (PHA) stimulated T cells and Epstein-Barr (EBV) virus transformed B cells from the same peripheral blood sample of a patient with ataxia-telangiectasia. We chose to study cells from a patient with this disease because of the predisposition of DNA from such patients to undergo breakage

events, thus, potentially increasing our yield of analyzable chromosomal abnormalities. Approximately 40% of the EBV transformed B cells from this patient were karyotypically normal except for a reciprocal translocation between chromosomes 2 and 14 with breakpoints within the bands in which the immunoglobulin kappa and heavy chain genes have been localized (7,8). We have single cell cloned this total B cell population and obtained a karyotypically normal and a t(2;14) clone for further analysis. By conventional analysis the t(2;14) clone has rearranged both mu genes, deleted both kappa constant regions but retained one rearranged kappa J segment linked to a kappa deleting element, rearranged two lambda light chain genes (the cell is a lambda producer) and deleted a particular D region family. We do not yet know whether the kappa deleting elements or the rearranged heavy chains are keys to the translocation.

The PHA stimulated T cells from this same patient did not show the t(2;14). Rather they showed translocations and inversions of chromosome 14 with breakpoints at 14q11.2 and 14q32. Thus, abnormalities were distinct in distinct cell types. B cells showed breaks in bands in which B cell differentiated functions were localized. Were the breaks seen in T cells from this patient relevant to T cell differentiated function? In collaboration with Dr. Tak Mak's lab we mapped the T cell antigen receptor (TCR) alpha chain gene to chromosome 14 band q11.2 (9). Thus, cell type specific chromosomal aberrations may direct attention to regions of differentiated functions within those cells. We presented and summarized these data in 1985 (10).

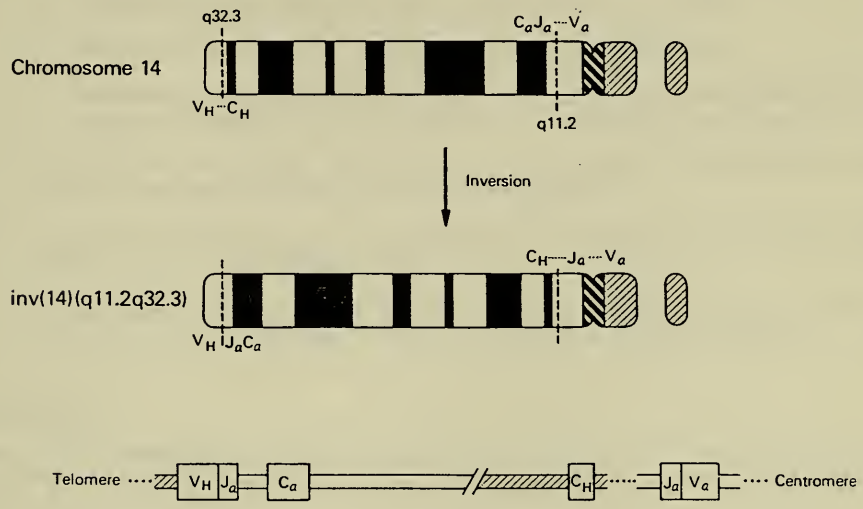
Molecular Cloning and Characterization of an Inversion of Chromosome 14

As is obvious from the previous sections we are interested in defining in molecular terms cell type specific chromosomal aberrations. With our identification of inversions and translocations in the T cells of the patient with ataxia-telangiectasia and our mapping of the TCR alpha chain gene to 14q11.2 we felt we potentially had a probe for analyses of some T cell specific chromosomal aberrations.

We received a cell line SUP-T1, derived by Dr. Stephen Smith from a 6 year old male hospitalized at Stanford Medical Center with T cell lymphoma. Karyotypic analysis by Drs. Frederick and Barbara Hecht had demonstrated an inversion of chromosome 14 in this patient's tumor cells; breakpoints were at 14q11.2 and 14q32. Using TCR constant and joining region probes, we were able to clone and sequence the breakpoints of this inversion. At the new 14q32 junction an immunoglobulin heavy chain variable region had become contiguous by site-specific recombination with a T cell antigen receptor alpha chain joining segment upstream of the TCR alpha constant region. The hybrid Ig variable-TCR joining segment had been formed in-frame and was transcribed into messenger RNA that include the TCR alpha constant region (11).

There has been some controversy concerning whether the same precise VJ recombination enzyme system can mediate the site-specific recombination event in both B cells and T cells, that is for both immunoglobulins and T cell antigen receptors. This example strongly suggests that not only can the same system recognize the relevant Ig and TCR signal sequences, it can unify them. As a corollary to this it must mean that these two loci, felt to be distinct and even differentially activated during lymphocyte development, must have been simultaneously accessible to the action of the same recombinase(s). This is also the best current example

where the occurrence of a particular chromosomal aberration can be modelled with perfect clarity. (Figure 3). One can envision each step in the process of the formation of this inversion as being analogous to normal VJ recombination. The ambiguity of the process here is only the ambiguity still limiting our understanding of the precise mechanistic features of VJ recombination.



The inversion of chromosome 14 in the SUP-T1 cell line.

This study suggests the possibility that early in lymphocyte development there might be simultaneous accessibility of Ig and TCR loci to a recombinase. This concept is strengthened by our analysis of a morphologically identical inversion of chromosome 14 in the tumor cells of an 18 month old female with CALLA positive ALL. The tumor is phenotypically and genotypically of B cell lineage. We have again cloned and sequenced the breakpoint of the inversion. Again it is a site specific recombination of an Ig heavy chain variable segment and a TCR alpha chain J segment; not the same V or J as in the SUP-T1 example described above, but precisely the same mechanism. The rearrangement has again occurred in-frame. Intact hybrid mRNA is again identified (12).

We feel that these studies speak to the nature of lymphocyte development and genomic accessibility. The occurrence of the inversion might also be relevant to the development of the malignant status of these two tumors in which it was studied. We are continuing to investigate both these issues.

Proposed Course

Our specific aims for the next segment of research in this project can be divided

into four categories reflecting the basic objectives we have stated in light of progress to date.

I. Follow-up work to our analyses of inversion (14) in two pediatric tumors.

   A. Analysis of Ig variable region transcription in T cells.

   B. Search for evidence of a hybrid Ig-TCR peptide product

   C. Analyses of the contributions of growth effecting genes (particularly c-myc and c-myb) to the development of hematopoietic malignancy.

II. The study of chromosomal aberrations in the "normal" or premalignant" B and T cells from patients with ataxia-telangiectasia.

III. The study of inversions and translocations of chromosome 14 in other T cell tumors, particularly prothymocytic CLL.

IV. The karyotypic and molecular analysis of tumor cells from the "mature" T cell tumors represented by mycosis fungoides and Sezary Syndrome.

Publications

Denny, C.T., Yoshikai, Y., Mak, T., Smith, S., Hollis, G.F., and Kirsch, I.R.: A chromosomal inversion in a T cell lymphoma is caused by site-specific recombination between immunoglobulin and T cell receptor loci. Nature 370:549-551, 1986.

Denny, C.T., Hollis, G.F., Hecht, F., Morgan, R., Link, M., Smith, S.D., and Kirsch, I.R.: Common mechanisms of chromosomal inversion in B and T cell tumors: Relevance to lymphocyte development. Science 234:197-200, 1986.

References

1. Lenoir, G.M., Preudhomme, J.L., Bernheim, A., and Berger, R.: Correlation between immunoglobulin light chain expression and variant translocations in Burkitt's lymphoma. Nature 298:474-476, 1982.

2. Denny, C.T., Hollis, G.F., Magrath, I.T., and Kirsch, I.R.: Burkitt lymphoma cell line carrying a variant translocation creates new DNA at the breakpoint and violates the hierarachy of immunoglobulin gene rearrangement. Molec. Cell. Biol. 5:3199-3207, 1985.

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9. Caccia, N., Bruns, G., Kirsch, I., Hollis, G., Bertness, V., and Mak, T.: The T cell receptor alpha chain genes are located on chromosome 14 at 14q11-14q12 in humans. J. Exp. Med. 161:1255-1260, 1985.

10. Kirsch, I.R., Brown, J.A., Lawrence, J., Korsmeyer, S.J., and Morton, C.C.: Translocations that highlight chromosomal regions of differentiated activity. Cancer Genet. Cytogenet. 18:159-171, 1985.

11. Denny, C.T., Yoshikai, Y., Mak, T., Smith, S., Hollis, G.F., and Kirsch, I.R.: A chromosomal inversion in a T cell lymphoma is caused by site-specific recombination between immunoglobulin and T cell receptor loci. Nature 370:549-551, 1986.

12. Denny, C.T., Hollis, G.F., Hecht, F., Morgan, R., Link, M., Smith, S.D., and Kirsch, I.R.: Common mechanisms of chromosomal inversion in B and T cell tumors: Relevance to lymphocyte development. Science 234:197-200, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06581-04 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Molecular Genetics of Differentiation and Transformation

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

PI:	W. Michael Kuehl, M.D.	Senior Investigator	NCI-NMOB
Others:	Timothy Bender, Ph.D.	Senior Staff Fellow	NCI-NMOB
	Shoshanna Segal, Ph.D.	Independent Invest., USUHS	NCI-NMOB
	Jeannine Stafford (term. 5/29/87)	Microbiologist	NCI-NMOB
	Maria Paez, Ph.D. (term. 5/31/87)	Visiting Fellow	NCI-NMOB
	Ethan Dmitrovsky, M.D. (term 6/6/87)	Biotechnology Fellow	NCI-NMOB
	Diana McClinton (started 6/8/87)	Microbiologist	NCI-NMOB

COOPERATING UNITS *(if any)*

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NCI-Navy Medical Oncology Branch

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Genetics, Molecular Biology and Immunology Section

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A. Overall objectives:

1. To clarify the cellular and molecular mechanisms which determine and regulate hematopoietic and stem cell differentiation.
2. To clarify the relationship between differentiation and malignancy.

B. Species studied: Mice and humans

C. Summary:

We have been studying the role of nuclear oncogenes in growth and differentiation. Much of our work has focused on c-myb which encodes a nuclear, DNA-binding protein. We have cloned and sequenced the mouse c-myb mRNA, and have defined the major transcription unit. The finding of 5' mRNA heterogeneity and different size forms of mouse c-myb protein suggests alternative 5' RNA splicing, although we have no definitive information to clarify this situation presently. Preliminary results indicate that the major mechanism by which c-myb expression is regulated in mouse hematopoietic cells is modulation of transcription elongation occurring at a site in intron 1. We have demonstrated that pre-B cell lines express 10-100 times as much c-myb mRNA as more mature B cells. This finding has helped us to demonstrate that pre-B cell lines which begin to express surface immunoglobulin retain a pre-B cell phenotype. Finally, we have established that down-regulation of c-myc mRNA is necessary for mouse erythroleukemia cells to undergo terminal differentiation; preliminary results suggest that down-regulation of c-myb mRNA is also necessary for terminal differentiation. These studies suggest that constitutive expression of normal proto-oncogenes may account for the observation that tumor cells are generally "frozen" in a particular state of differentiation.

PROJECT DESCRIPTION

Molecular Genetics of Differentiation and Transformation

1. STRUCTURE, EXPRESSION, AND FUNCTION OF MURINE c-myb PROTO-ONCOGENE

Professional Staff:

PI:	W. Michael Kuehl, M.D.	Senior Investigator	NCI-NMOB
Others:	Timothy Bender, Ph.D.	Senior Staff Fellow	NCI-NMOB
	Jeannine Stafford (term. 5/29/87)	Microbiologist	NCI-NMOB
	Diana McClinton (started 6/8/87)	Microbiologist	NCI-NMOB

Three kinds of approaches have been used (i.e., tumor cell lines, induction of differentiation in a cloned cell line, and somatic cell hybrids) to study differential expression of c-myb proto-oncogene mRNA in murine B cell tumors. To summarize, in murine B-cell tumor lines and hybrids, the level of c-myb mRNA expression reflects the level of B-cell differentiation. The results obtained for the line that differentiates are also consistent with the concept that c-myb expression is markedly downregulated (ten-fold or more) as B cells mature beyond the pre-B cell stage of development. Since all studies were done with exponentially growing cells, the down-regulation of c-myb clearly correlates with differentiation and not with changes in cell proliferation.

We have determined the DNA sequence of murine c-myb mRNA. Starting with an avian V-myb probe we initially isolated genomic clones covering 30 Kb, including sequences at each end of this span which hybridize to c-myb mRNA but not to a V-myb probe. We prepared a murine pre-B cell cDNA library, and isolated ten c-myb cDNA clones. Together two of our cDNA clones include approximately 3.4 kb of c-myb mRNA sequence. The sequence of these clones, together with S1 nuclease protection studies, have defined the murine c-myb transcription unit. In the course of these studies we have found an extreme degree of heterogeneity of the 5' end of murine c-myb mRNA. We are attempting to clarify the biological significance of this finding. We have begun to extend our studies to the human c-myb gene. To assess potential 5' regulatory sequences we sequenced the 5' end of the coding region plus about 1.5 Kb of flanking region of human c-myb genomic clones; there is striking homology to the mouse gene even in 5' flanking sequences. Recently we have demonstrated that a major mechanism regulating the level of c-myb mRNA expression in murine B cell lines is a block to transcription elongation (? pausing or ? premature termination) occurring near the middle of intron one, a genomic region which contains a more predominant DNaseI hypersensitive site in a B cell line (A20/2J) compared to a pre-B cell line (70Z/3B). We are also studying the structure and expression of the c-myb gene in a small cell lung cancer line which has amplified the c-myb gene. Finally we are beginning to: 1) express c-myb in yeast in order to prepare antisera and to provide large amounts of c-myb protein for study, 2) transfect c-myb expression vectors into various eukaryotic cells, and 3) look for c-myb gene rearrangements and amplifi-

cation in other human tumors. Our long term goal is to determine the role of the c-myb proto-oncogenes in hematopoietic differentiation and tumor induction.

Publications:

Bender, T.P. and Kuehl, W.M.: Structure and expression of c-myb proto-oncogenes mRNA in murine B cells. Curr. Topics Microbiol. and Immunol. 132: 153-157, 1986.

Bender, T.P. and Kuehl, W.M.: Murine myb proto-oncogene messenger RNA: cDNA sequence and evidence for 5' heterogeneity. Proc. Natl. Acad. Sci. 83: 3204-3208, 1986.

Bender, T.P. and Kuehl, W.M.: Structure and differential regulation of the myb proto-oncogene in murine B lymphoid tumors. In Cellular and Molecular Biology of Tumor and Potential Clinical Applications. New York, Alan R. Liss, Inc. (In press).

Bender, T.P. and Kuehl, W.M.: Differential expression of the c-myb proto-oncogene marks the pre-B/B cell junction in murine B lymphoid tumors. (Submitted to J. Immunology).

Bender, T.P., Thompson, C., and Kuehl, W.M.: A block to elongation in the first intron of c-myb is responsible for regulating transcription levels in murine B lymphocytes. (Submitted to Science).

2. ROLE OF c-myc AND OTHER ONCOGENES IN DIFFERENTIATION (in collaboration with G. Hollis, I. Kirsch).

Professional Staff:

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	Shoshanna Segal, Ph.D.	Independent Invest., USUHS	NCI-NMOB
	Jeannine Stafford (term. 5/29/87)	Microbiologist	NCI-NMOB
	Maria Paez, Ph.D. (term. 5/31/87)	Visiting Fellow	NCI-NMOB
	Ethan Dmitrovsky, M.D. (term. 6/6/87)	Biotechnology Fellow	NCI-NMOB
	Diana McClinton (started 6/8/87)	Microbiologist	NCI-NMOB

Endogenous c-myc and c-myb mRNA levels decrease rapidly when murine erythroleukemic (MEL) cells are induced to differentiate with DMSO. We have introduced a c-myc construct into MEL cells by stable transformation in order to determine whether constitutive expression of myc mRNA blocks differentiation or inhibits expression of endogenous myc mRNA. The expression of exogenous human c-myc mRNA blocks DMSO-induced differentiation of MEL cells. In addition, expression of endogenous c-myc mRNA is not turned off by exogenous c-myc, although the level of expression of the endogenous c-myc is inversely proportional to the level of expression of exogenous c-myc in the first five stable transfectants analyzed. Studies to determine the possible molecular basis for these results are in progress. Preliminary results indicate that MEL cells which constitutively express an exogenous c-myb construct are also blocked in their ability to terminally

differentiate when an appropriate inducer is added. We are now introducing N-myc into these cells to determine if a myc family gene which is not expressed in MEL cells can also block DMSO-induced differentiation. In addition, studies in F-9 teratocarcinoma cells indicate that the block of differentiation by constitutive c-myc expression is general.

Publications:

Dmitrovsky, E., Kuehl, W.M., Hollis, G.F., Kirsch, I.R, Bender, T.P. and S. Segal. Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukemia cell line. Nature 322:748750, 1986.

Dmitrovsky, E., Kuehl, W.M., Hollis, G.F., Kirsch, I.R., Bender, T.P. and Segal, S.: A transfected c-myc oncogene inhibits mouse erythroleukemic differentiation. Curr. Topics Microbiol. and Immunol. 132: 327-330, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06587-03 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Tumor-Specific Gene Alteration and Expression

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

PI:	I.R. Kirsch, M.D.	Senior Investigator	NCI-NMOB
Others:	N. Seibel, M.D.	Medical Staff Fellow	NCI-NMOB
	C. Felix, M.D.	Medical Staff Fellow	NCI-NMOB
	V. Bertness	Biol. Lab Tech	NCI-NMOB
	K. Nakahara	Bio. Lab Tech	NCI-NMOB

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NCI-Navy Medical Oncology Branch

SECTION

Genetics, Molecular Biology & Immunology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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

☐ (a1) Minors

☐ (a2) Interviews

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

Differential structural alterations and expression of immunoglobulin (Ig), T cell receptor (TCR), and various growth effecting genes are studied in malignant tumors and their derivative cell types. Studies are carried out to diagnose, classify, and stage lymphoid malignancies via a) Southern and Northern blot analysis and b) RNA-RNA tissue in situ hybridization.

a) DNA and RNA is extracted from the tumors of patients with acute lymphoblastic leukemia (ALL) of infancy, pre B ALL, T cell ALL, mycosis fungoides, and Sezary syndrome. The structural reconfigurations of DNA around the Ig and TCR loci resulting from the normal functional activation of these loci in these cells provide unique "fingerprints" for identifying clonal populations in the samples and following these populations during the course of treatment. We have shown by these analyses that the above listed lymphoid malignancies each manifest generally distinguishing genotypic patterns reflecting target cell maturation which to a certain extent recapitulates the age incidence of the development of these tumors.

b) RNA-RNA tissue in situ hybridization. The expression of individual cells within tissue sections from lymph node biopsies and peripheral blood from patients with lymphoid malignancies have been analzyed with immunoglobulin, T cell receptors, and oncogene probes. This technique refines the analysis of such tissue to the point where the unique gene expression of one cell in hundreds of thousands can be identified. Applying this technique to patients with CLL has revealed an unexpected cellular heterogeneity in the involved tissues. Analyses of patients with Hodgkin's disease is beginning to shed light on the origin and role of the different cell types (including the Reed-Sternberg cell) in this disease.

The research and clinical utility of this technique is profound and it can be as easily and systematically applied as are current histochemical and immunocytochemical techniques.

PROJECT DESCRIPTION

Tumor-Specific Gene Alteration and Expression

Professional Staff:

PI:	I.R. Kirsch, M.D.	Senior Investigator	NCI-NMOB
Others:	N. Seibel, M.D.	Medical Staff Fellow	NCI-NMOB
	C. Felix, M.D.	Medical Staff Fellow	NCI-NMOB
	V. Bertness	Biol. Lab Tech	NCI-NMOB
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Objectives

1. To develop, master, and refine techniques based on molecular genetics which are of direct current application in the diagnosis, classification, and staging of patients with cancer.

2. To demonstrate the usefulness of these techniques in pilot studies.

3. To promote the adoption of these techniques by service oriented laboratories, and supervise the implementation of such techniques in a standardized quality controlled fashion for comprehensive, prospective, best available therapy protocols and epidemiological studies.

4. To use the data generated in the pilot and/or comprehensive analyses as a resource of information and a bank of material for further studies to be carried out in basic research laboratories.

Methods Employed

As a result of the normal functionally activating recombination events that occur in B and T lymphocytes, the structural configuration of the DNA around the immunoglobulin or T cell receptor loci is irreversibly altered in each differentiated cell. Because of the unique nature of the antigen-receptor molecules being produced, the VJ or VDJ recombinatorial event (and DNA configuration) in one cell making one antibody or T cell receptor will be distinct from a cell making an antibody or T cell receptor with a different antigen-binding capacity. Thus, by the necessity of its role in the immune response, every lymphocyte beyond a certain stage in its development carries within it a unique molecular DNA "fingerprint".

A B or T lymphocytic malignancy results in the clonal proliferation of a cell that therefore can carry a unique molecular fingerprint. Recombinant DNA technology now allows the recognition of this fingerprint when it is present in 0.1% to 1% of a total cell population. Therefore, molecular biology can increase the sensitivity of tumor detection in a tissue sample. It can classify tumors as being of T or B cell lineage, identify biclonal tumor populations, distinguish new primary lymphomas from relapsed cases, and address basic questions of tumor development and progression. This basic molecular technology can now be success-

fully combined with fine needle aspiration of lymph nodes to aid in the diagnosis and staging of patients with lymphomas. It may very likely become a routine test in the general work-up of patients with lymphoma at presentation and at relapse. There is an essential need to incorporate the information that will be generated from these tests into prospective clinical trials. After these trials it can be anticipated that routine management of patients with lymphoma will be influenced by molecular data as well as the other currently available analyses.

This technology is not restricted to use in the leukemias and lymphomas. Although the normal functional rearrangements that these cells undergo provide a special feature for studying lymphoid malignancies the molecular biological techniques are of general use. It is possible that gene systems in other cell types will undergo similar DNA configuration-altering recombination events as part of their functional development. There is evidence that immunoglobulin-like recombination signals exist outside of the immunoglobulin loci themselves, possibly in other gene systems. Of more likely usefulness, however, will be the ability to monitor DNA rearrangements and amplifications that are directly related to the development of a cancer and not just markers of the transformed cell type. This is a likely possibility as more and more specific cancers are being shown to be associated with distinctive chromosomal aberrations: translocations, amplifications, deletions, or inversions. On the level of the DNA, these aberrations often result in the development of distinguishing new configurations of DNA (a tumor-specific molecular fingerprint) often related to oncogenes; i.e. genes that are thought capable of contributing to the development of a malignant phenotype in a normal cell. Thus, a wide variety of tumors are approachable using the same basic molecular technology. In many cases these chromosomal aberrations contribute to the etiology of cancers by altering the quality or quantity of RNA transcribed and translated from those genes structurally flanking the chromosomal aberrations. It is now possible to identify (at the level of individual cells within histologic tissue sections) those cells that are expressing a particular gene or genes. This technique is called RNA-RNA tissue in situ hybridization. Our experience with this technique will be outlined later in this report. Thus, technically, the potential exists to redefine histopathologic diagnoses on the basis of particular gene expression within tumor cells.

Over the next 5 years pathologists may begin reporting information about rearrangements, amplifications, and aberrant expression of various oncogenes in tumor biopsy specimens which an oncologist will then use to define prognosis or design a regimen of therapy. For the practicing oncologist, the molecular causes and consequences of a patient's disease should become relevant to determining that patient's diagnosis, course, and treatment. In the next few pages we will describe early explorations we have made as well as ongoing work and future plans directed toward the goal of hastening the inclusion of molecular genetic technology in the workup of patients with cancer.

Major Findings

Patient Studies

The ability to detect immunoglobulin (Ig) and T cell antigen receptor (TCR) gene rearrangements is proving useful in confirming diagnosis of suspected B or T cell lymphoid malignancies and in establishing mono, oligo,

and polyclonality in these and possibly related disorders.

Mycosis fungoides, Sezary syndrome, T-gamma lymphocytosis

In an early study we employed cloned probes for the TCR loci and Southern blot analysis (see references to basic molecular biological techniques at the end of reference list) to determine whether gene rearrangements were present in human T cell neoplasms representing various stages of T cell development (1). Gene rearrangements were present in all cases of immunophenotypic T cell disorders except a single case of T gamma lymphocytosis. Subsequent work by us and others have shown this disorder to be variable in terms of identification of clonal TCR rearrangements possibly reflecting disease heterogeneity and/or temporal evolution of clonality during the disease process. Germline gene configurations were present in all patients with "mature" non T cell neoplasms and in uninvolved tissues from patients with T cell lymphoma.

Acute T cell ALL of childhood

TCR and Ig genes were examined in 26 cases of childhood T-ALL and 17 cases of pre B ALL (2). TCR gamma was rearranged in 22 of the 26, TCR beta in 23 of the 26, one or the other or both in almost every case of phenotypic T-ALL. Rearrangement of both alleles of the gamma and beta chains occurred in most T-ALLs. Expression of the beta and/or gamma chains was observed commonly in these tumors. Alpha chain gene expression was found less often, usually only in the most mature T-ALL's which were T3+. Analogous to studies of pre-B ALL (3) molecular analysis of T-ALL suggests a hierarchy of early gamma and beta gene rearrangement followed by alpha, and a coordinate sequence of early appearance of the 3A1 antigen, followed by T11 and later T3. Three patients in this series with T-ALL also showed evidence of Ig heavy chain gene rearrangement. Of the 17 patients with pre-B ALL eight showed rearrangements of the TCR gamma gene. Evidence of activity of these two loci, felt to encode distinct differentiated functions, is much more commonly seen in these tumors of lymphocytes of less mature stages of development than in the more mature adult tumors such as those mentioned earlier. This may speak to an interesting issue of lymphocyte development which has been addressed in a separate project report.

Acute ALL of infancy

ALL in infants less than one year of age runs a particularly virulent course. It, therefore, was of interest to genotypically analyze a group of these patients to possibly contrast their patterns of gene rearrangement with those seen in older children as described above, as well as those seen in adults (4) (See Table 1). Lymphoblasts of 11 infants demonstrated surface antigens which have been correlated with a pre-B cell phenotype. 4/11 of these retained the germline configuration of both Ig and TCR genes, suggesting that ALL in infancy represents an earlier stage of B cell development than is found in pre-B ALL of older children, where all had rearranged at least Ig H-chain genes. No phenotypic TALL patients were found in this study. As mentioned above, Ig H-chain rearrangements were occasionally seen in the series of T-ALL's, and TCR gene rearrangements, especially gamma, were identified in a large proportion (45%) of pre-B ALL's of older children. In contrast, TCR gamma gene rearrangements were not identified in pre-B ALL's of infants. These studies extend the earlier studies of pre-B ALL

Table 1. Genotypes and Phenotypes of Infant ALLs

Pt.#	Ig H-Chain		k L-Chain		TCR	TCR		HLA-DR	B4	CALLA	SIg	3A1	T11	L4F3	Ig10/ 5F1	13.1	C7E10	12-8
1	R	1	G		G	G		+	+	-	-	-	-	-	-	-	-	17
2	R D	1 1	G		G	G		+	+	-	-	-	-	-	-	-	-	-
3	R	2	G		G	G		+	+	15	-	-	-	-	-	-	-	-
4	G		G		G	G		-	32	36	-	-	-	-	-	-	20	-
5	R	2	R	1	G	G		+	+	-	-	-	-	12	-	-	-	-
6	G		G		G	G		20	11	+	-	-	-	-	-	-	-	-
7	R	1	R	1	G	R	1	+	+	11	-	-	-	-	-	-	-	-
8	G		G		G	G		+	+	-	-	-	-	-	-	-	-	-
9	R	1	G		G	G		+	+	-	-	-	-	-	-	-	-	-
10	R	2	G		G	G		+	+	-	-	-	-	-	-	-	-	-
11	G		G		G	G		+	+	-	-	-	-	-	-	-	-	+

Legend: Pt.=Patient, R=Rearranged, D=Deleted, G=Germline, No.=Number of alleles, (-) is <10% of cells showing reactivity, (+) is >50%, and specific values (%) are given for the 10-50% range. Phenotypic data (except for SIg and 3A1) were previously tabulated in Dinndorf and Reaman (16). The germline configuration of the Ig H-chain locus was confirmed for patients 4 and 11 using HindIII and/or EcoRI digests in addition to BamHI digests probed with the $3'J_H$ probe. The germline configuration of the γ TCR locus was found in every case by probing BamHI digested DNA with the $C_T\gamma$, $J\gamma_{1,3}$, and an entire TCR γ cDNA, and further confirmed by hybridization of EcoRI blots with the $J\gamma_{1,3}$ rppobe for patients 4, 5, 10, and 11. The δ TCR locus was studied by hybridization of BamHI digested DNA with the $C_T\delta$ probe in all cases, and hybridization of EcoRI digested DNA with the same probe for patients 4, 5, 10, and 11.

in older children. Together they suggest discrete stages in lymphoid development vulnerable to malignant transformation. Because the distribution of ALL subtypes throughout childhood is not uniform, these data suggest either a change in size of the target cell population, or a differential vulnerability of lymphocyte subpopulations to etiologic agents with increasing age.

Family studies

Insertions, deletions, amplifications, and point mutations of genes occur in DNA throughout the evolutionary process. When these events occur in germline DNA, they are transmitted vertically from one generation to the next. Differing mutation sites and mutation rates between species and within a given species can be identified by comparison of defined populations by restriction enzyme site patterns for given probes of interest. This study of "restriction fragment length polymorphisms" ("RFLPs") reflecting the structural variability of DNA can be utilized in evolutionary, population, and family studies. Unlike the rearrangements of Ig or TCR genes, these RFLPs need not indicate the programmed rearrangement of a particular locus during the ontogeny of a particular cell type. Molecular genetic technology of DNA analysis can still be applied to this study. In what follows we describe one such example of RFLP analysis in which we have been involved. Its significance to the occurrence of a particular tumor in a particular family remains to be seen. Conceivably it could provide a marker for disease risk in this case. It would also be suggesting a mechanism of tumor formation akin to those now postulated, for example, for Wilm's tumor or retinoblastoma. We investigated (5) a family in which a father and three of his offspring had meningioma with clinical onset at the ages of 35 to 65 years. A fourth offspring died of multiple neoplasms arising at 29 years. No one in the family had any evidence of von Recklinghausen disease. The three siblings with meningioma carried a constitutional Robertsonian translocation, t(14;22) (14qter cen 22qter), in peripheral blood leukocytes. Three other members of the second generation who were beyond the age at which the onset of meningioma is expected had no tumors and had normal karyotypes. In the third generation, whose members are now reaching the age for tumor onset, four carriers of the translocation have been identified; to date they are all asymptomatic except for one woman, who has breast cancer.

Both living members with meningiomas had a polymorphic variant of the c-sis oncogene in peripheral-leukocyte DNA, according to analysis with the Southern blot technique. This variation was also present in one asymptomatic member of the third generation and segregates with the morphologically normal No. 22 chromosome in both the affected and nonaffected members. It is possible that an "active" or mutant gene on the long arm of chromosome 22, possibly even the c-sis oncogene itself may be associated with the development of meningiomas in this kindred. Analysis of the meningioma tumor tissue itself would be of great interest in this regard but is presently not available. Sporadic meningioma is known to often to be associated with monosomy of chromosome 22 or less frequently the absence of the long arm of chromosome 22 (6). The c-sis proto-oncogene has been localized to the tip of the long arm of chromosome 22 (7).

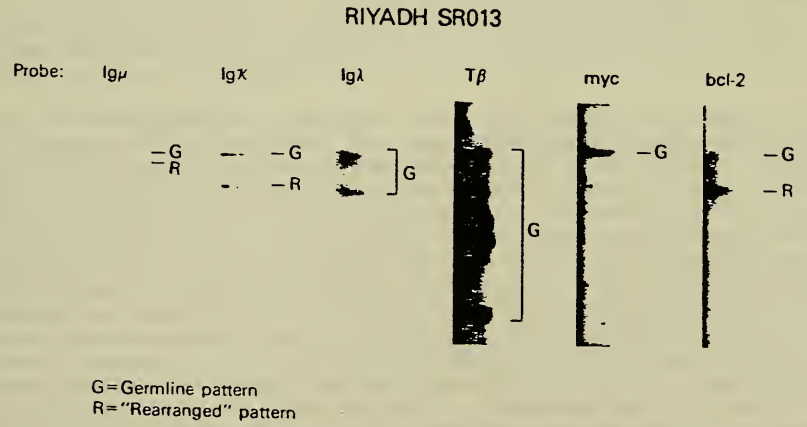
Epidemiological Studies

We carried out a pilot feasibility study in collaboration with Dr. Ian Magrath

of the Pediatric Branch, National Cancer Institute, USA and Dr. Gregory O'Conor, then Senior Scientist, International Agency for Research on Cancer, Lyon, France, to determine whether the logistics of tissue collection from widely dispersed and varied patient care centers could be set up so as to allow for appropriate and reproducible analysis of DNA rearrangements.

The most basic questions remain unanswered concerning worldwide presentation and distribution of different types of lymphoid leukemia and lymphoma. The initial screening assay on the patient samples was a determination of TCR beta and Ig heavy chain gene rearrangements. Samples were collected from Nagoya and Kyoto, Japan, from Riyadh, Saudi Arabia, from Lima, Peru, from two centers in Budapest, Hungary and from New Delhi, India and sent to us via Lyon, France. We were easily able to study rearrangements of Ig and TCR loci in most of the samples received. Thus, it is reasonable to consider such international molecular genetic epidemiological studies. For a selected number of samples we extended our analysis to include studies of kappa and lambda light chain gene rearrangement and rearrangement of a select number of proto-oncogenes.

The ultimate goal of such an epidemiologic study would be to correlate the specific type of lymphoma with geographical distribution. In its most fundamental form this would involve relating the frequency of a specific Ig or TCR gene rearrangement and state of maturity of the tumor cell to the distribution of these malignancies in different parts of the world. Ultimately, the survey would be expanded to include information on oncogene expression and association with specific viruses. One need only consider how the recognition of endemic Burkitt's lymphoma in equatorial Africa or HTLV-1 positive acute T cell leukemia in the southern provinces of Japan or the Caribbean has been of such crucial importance for basic research as well as patient diagnosis and treatment. Often on the basis of DNA analysis alone one is able to make significant progress toward patient diagnosis and classification. An example of this is shown in Figure 1.



Genotypic analysis of a lymph node sample from a patient from Riyadh, Saudi Arabia.

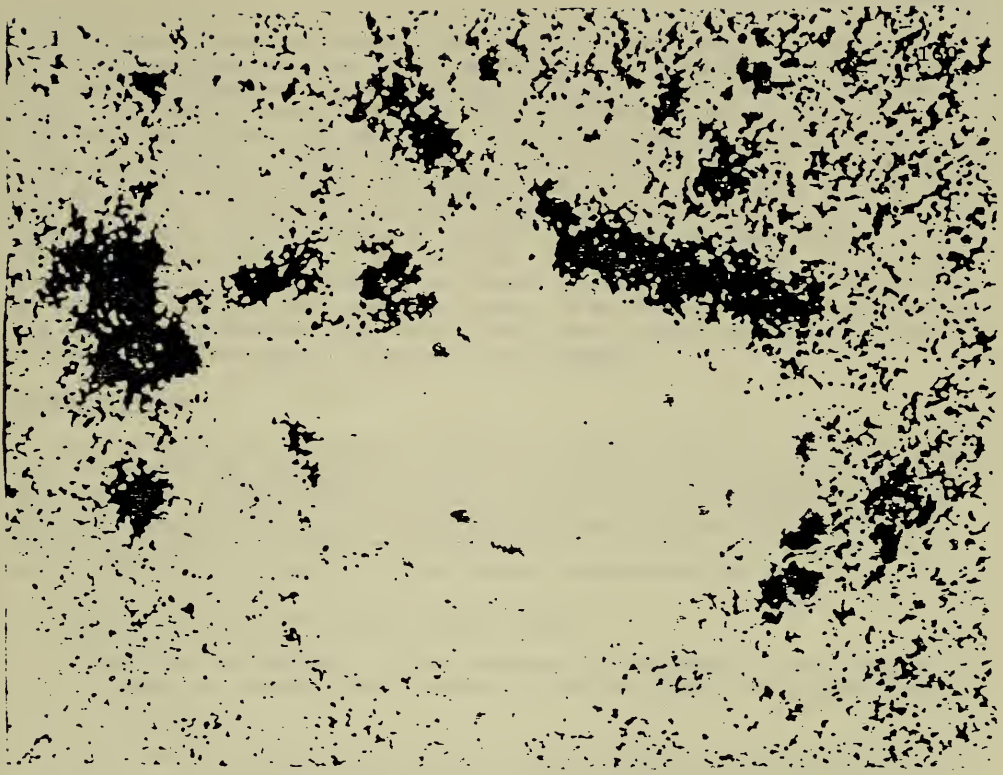
On a sample of lymph node from Riyadh, Saudi Arabia for which no clinical or laboratory data were available, we were able to discern a rearranged Ig heavy chain gene and kappa light chain gene, germline Ig lambda, TCR beta, and c-myc genes and a rearranged bcl-2 gene. On the basis of this analysis alone, it would be highly likely that such a patient suffered from a B cell follicular lymphoma with a t(14;18) translocation.

RNA-RNA tissue in situ hybridization

As described in the previous section (as well as in the work of dozens of laboratories all over the world) multiparameter studies including cytochemistry, ultrastructural analysis, immunophenotying, and recombinant DNA analyses of nucleic acids have contributed greatly in recent years to the classification and subclassification of lymphoid malignancies and other tumors. The kinds of recombinant DNA analyses described above examine the entire cell population, and thus the data represent an average of gene rearrangement or expression in which heterogeneity in the tumor can only be inferred. Recently it has become possible to use cloned nucleic acid probes at the level of individual cells in tissue sections (8). In conjunction with routine histologic diagnosis, this technique holds the promise of directly assessing cellular heterogeneity in a tumor, classifying tumors by their unique patterns of gene expression, and potentially gaining insight into etiologic and developmental questions in the disease state being analyzed.

As an early step in our utilization of this technique, we wished to establish the credibility of the data we could generate with it, with reference to the conventional techniques of molecular genetic analysis with which we were familiar and comfortable. We, therefore, conducted an analysis of an involved lymph node and peripheral blood from a patient with lymphadenopathy and a elevated white count. DNA and RNA from these tissues were analyzed by Southern and Northern blot, respectively, using ^{32}P probes derived from the Ig heavy and light and TCR beta chain loci. ^{35}S complementary RNA probes from these same loci were applied to fixed sections of these tissues and (after appropriate hybridization and washing conditions) autoradiographed.

We found the different techniques to be confirmatory and complementary (9). The technique is also consistent with immunophenotypic data generated on the same tissue. Our studies demonstrate that this patient suffered from a B cell malignancy. His lymph node showed a monoclonal B cell population and a polyclonal T-cell infiltrate. In his peripheral blood only the monoclonal B cell population was seen. Furthermore, in the malignant B cell population a provocative cellular heterogeneity was noted, a heterogeneity that showed an interesting distribution pattern within the lymph node studied. There was a diffuse clonal infiltrate of B lymphocytes expressing Ig mu and lambda message. Clustered around the vessels, however, were encountered apparently the same clonal population but with a much increased expression of the Ig mRNAs (see Figure 2). The data and observations derived from this study supported our expectations that, when combined with clinical observations and other methods in histopathology, this kind of analysis could provide a general tool for answering important diagnostic and prognostic questions.



RNA-RNA tissue in situ hybridization using a Ig mu cRNA probe on a lymph node from a patient with chronic lymphocytic leukemia.

With this in mind we applied this technique to a study of myc related (c-myc, N-myc, L-myc) proto-oncogene expression in small cell lung cancer (10). The tissues investigated included cytospins of ten cell lines derived from patients with SCLC, four corresponding nude mouse xenografts from cell lines, and metastatic tumor tissue obtained by surgical biopsy and at autopsy. The expression of each gene was specifically demonstrated by autoradiography in the cytoplasm of the neoplastic cell samples. The average levels of oncogene expression in each specimen corroborated previous data obtained by Northern blot assay. In addition, heterogeneity in gene expression from cell to cell in each sample was noted. This study represented the first attempt to demonstrate oncogene expression in lung cancer cells in situ, and confirmed that the expression of these myc related genes can be seen in the primary tumor.

Chromosome in situ hybridization

Our group was one of those early involved in the localization of genes in the human genome by the technique of chromosome in situ hybridization following the technique first developed by Harper and Saunders (11). Since our first effort localizing the Ig heavy chain locus to 14q32 (12), we have conducted numerous

other studies. These studies have included the localization of c-myc to 8q24 (13), the localization of the beta globin cluster to 11p15 (14, in collaboration with Dr. Cynthia Morton) the localization of L-myc to 1p32 and of an amplified c-myc to an HSR in a small cell lung cancer cell line (in collaboration with Dr. John Minna and Dr. June Biedler). We have localized the human beta 1-4 galactosyltransferase to 9p13 (within the same chromosome band as is found in the gene defective in the disease galactosemia) (15). In collaboration with Dr. Tak Mak we localized the gene for the TCR alpha chain locus to 14q11.2 (16). In collaboration with Dr. James Battey we localized the gene for human gastrin releasing peptide to 18q21 (17). This was the same site to which we, in collaboration with Dr. Stan Korsmeyer, and others (18), had localized the bcl-2 gene. We have also utilized this technique as part of our molecular genetic analyses of the human T cell lymphoma line SUP-T1, and a human myeloma cell line H929 (described in a separate part of the site visit report).

Proposed Course

Our laboratory has established itself as having expertise in the basic techniques of DNA and RNA isolation and analysis, RNA-RNA tissue in situ hybridization, and chromosome in situ hybridization. Our specific aims and future plans fall into two categories, those that would be undertaken in-house primarily by the limited personnel of our own research laboratory, and those in which we would wish to participate as part of a much larger comprehensive study.

I. RNA-RNA tissue in situ hybridization studies

A. Analyses of testicular biopsy specimens for evidence of tumor cell infiltrate, at presentation or relapse in children with acute lymphoblastic leukemia.

B. 1. Direct RNA sequencing of Ig or TCR variable regions from leukemic cells. Synthesis of complementary oligonucleotide probes and the use of these probes in tissue in situ hybridization as tumor specific markers for clinical staging.

2. Direct RNA sequencing of mutable regions of expressed oncogenes from specific lymphoid and solid tumors. Depending on the extent of mutation, synthesis of complementary oligonucleotide probes for use as tumor specific markers as in B1.

C. Analysis of "small round cell tumors" of childhood with an attempt to develop a classification scheme based on distinctive gene expression within individual tumor cells.

II. Tumor genotyping service

The establishment of a service facility for processing of tissue samples, and DNA and RNA preparation. The capacity to comprehensively screen selected patient populations or tumor samples for the rearrangement and/or expression of particular genes of interest.

Publications

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Lebacq-Verheyden, A.M., Bertness, V., Kirsch, I., Hollis, G.F., McBride, O.W., and Battey, J.: Human gastrin-releasing peptide gene maps to chromosome band 18q21. Somatic Cell Molec. Genet. 13:81-86, 1987.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06589-03 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Biology and in vitro growth and drug sensitivity testing and colorectal carcinomas

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

PI:	Adi F. Gazdar, M.D.	Senior Investigator	NCI-NMOB
Others:	Ilona Linnoila, M.D.	Senior Investigator	NCI-NMOB
	Daniel Ihde, M.D.	Senior Investigator	NCI-NMOB
	James Mulshine, M.D.	Senior Investigator	NCI-NMOB
	Bruce Johnson, M.D.	Senior Investigator	NCI-NMOB
	Barnett Kramer, M.D.	Senior Investigator	NCI-NMOB
	John Minna, M.D.	Chief	NCI-NMOB

COOPERATING UNITS *(if any)*

Pediatric Oncology Branch, Medicine Branch, Clinical Pharmacology Branch, Radiation Oncology Branch

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Human Tumor Biology Section

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	5	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.)*

Summary of work

A) We have analyzed the histopathology of the first 100 cases entered onto the NSCLC protocol (83-15). These studies indicate a continuation of a trend noticed earlier, namely a dramatic absolute and relative decrease in the incidence of squamous cell carcinoma, and an absolute and relative increase in the incidence of adenocarcinoma, espeically bronchioloalveolar carcinoma (BAC).

B). We have develped methods and media for the selective growth of lung and colorectal carcinomas. With these techniques, our successful culture rates (per adequate tumor containing sample) are as follows: Small cell lung cancer (SCLC) 46%; non-SCLC (NSCLC) 23%; colorectal carcinoma 35%.

C). Currently, 48 NSCLC lines have been established and partly or completely characterized. They include several unique lines including mucoepidermoid, BAC and carcinoids. The BAC lines include several releasing large amounts of fully processed surfactant, and are being explored for use in the respiratory distress syndrome of neonates.

D). About 12% of NSCLC tumors express neuroendocrine properties similar to SCLC. We have established 5 lines from these tumors. Their in vitro chemosensitivity profiles are similar to those of SCLC cell lines and different from the relatively resistant profiles of NSCLC lines. These data suggest that a subset of NSCLC tumors (13000 cases/year) may be relatively chemosensitive.

We have modified a semiautomated (MTT) dye assay for in vitro chemosensitivity testing. The test is highly reproducible. Extensive testing of lung and colorectal cell lines suggest that in vitro testing may have relevance as a drug prescreen as well as for individualized selection of chemotherapy.

PROJECT DESCRIPTION

Biology and in vitro growth and drug sensitivity testing and Colorectal Carcinomas

Professional Staff:

PI:	Adi F. Gazdar, M.D.	Senior Investigator	NCI-NMOB
Others:	Ilona Linnoila, M.D.	Senior Investigator	NCI-NMOB
	Daniel Ihde, M.D.	Senior Investigator	NCI-NMOB
	James Mulshine, M.D.	Senior Investigator	NCI-NMOB
	Bruce Johnson, M.D.	Senior Investigator	NCI-NMOB
	Barnett Kramer, M.D.	Senior Investigator	NCI-NMOB
	John Minna, M.D.	Chief	NCI-NMOB

Objectives

One of the major objectives of this Branch is to develop newer, more rational therapies for the cancer types representing our major clinical interests. By studying the biology of specific cancers in depth, new ideas for cancer control are generated, tested in vitro and brought to clinical trial. We presume that such an approach is more likely to lead to advances in the therapy of refractory tumors such as colon and non-small cell lung cancer (NSCLC) than the development of new nontargeted cytotoxic agents. Further, a comprehensive knowledge of the biology of a cancer type can aid the physician in interpreting certain linical phenomena such as hormone secretion, tumor progression, etc. Finally, identification of tumor markers may aid diagnosis, staging, detection of relapse, imaging, subtyping, and monitoring response to therapy.

Three of the currently active clinical protocols for the therapy of SCLC and NSCLC depend on the selection of individualized patients' chemotherapy by in vitro drug sensitivity testing. Thus one of the major objectives of the Branch is to develop methods to (1) amplify tumor cells so that adequate numbers are available for testing; (2) develop and apply rapid, accurate, reproducible testing procedures; (3) demonstrate the clinical relevance of the testing procedures; and (4) utilize in vitro testing for biological and preclinincal studies.

About 12 years ago we began an in depth investigation into the biology of small cell lung cancer (SCLC), which was our major clinical interest at that time. This effort has gradually expanded to involve many investigators and much of the Branch's lab efforts. Partly as a result, as well as from the work of others, we have amassed considerable information about the biology of SCLC. These advances include identification of the basic growth factor requirements, establishment and continuous growth of SCLC in defined media, identification of

SCLC as a typical neuroendocrine (NE) tumor, recognition of specific peptides associated with SCLC, especially bombesin/gastrin releasing peptide (BN/GRP), identification of BN/GRP as an autocrine growth factor; identification of a specific chromosomal abnormality deletion 3p, and identification and characterization of the variant subtype of SCLC and its frequent association with amplification of the c-myc gene. Many recent developments are described elsewhere by other investigators. Multiple cell lines have been distributed to over 100 investigators throughout the world.

Some of our new discoveries have had clinical applications. Multiple markers are used to distinguish SCLC from routine NSCLC tumors, as well to identify a subset of NSCLC tumors with NE markers. The latter, referred to as NSCLC-NE, are being identified prospectively and treated with a SCLC-like chemotherapy regimen. BN/GRP is being investigated as a clinical marker in CSF and urine. Antibody to BN, 2A11, is being tested in a phase I trial. Our ability to reproducibly culture SCLC tumors forms the heart of the trials based on in vitro testing.

While continuing our studies with SCLC, we launched a similar targeted approach to the study of NSCLC about 3 years ago. NSCLC constitutes 75% of lung cancer and is relatively resistant to chemotherapy. Of particular interest, we have identified an important subgroup of NSCLC tumors which express SCLC-like markers. These findings, in addition to being of possible clinical relevance, indicate the inter-relationships and possible common origin of all lung cancers.

Recently, we have initiated similar pilot studies into the biology of colorectal carcinomas. Colorectal tumors are the second commonest cause of cancer in the USA and are highly resistant to chemotherapy. We have no current clinical protocols for this disease. However, in the anticipation that such studies will be initiated in the future, we have started studying this disease.

For more than 30 years attempts have been made to individualize therapy based on in vitro testing. The development and promotion of clonogenic assays in semi-solid media provided a major impetus for these studies. The major advantage of clonogenic assays is the selective growth of tumor cells. The major disadvantages include difficulties in preparing true single cell suspensions, low clonogenic potential of many human tumors and cell lines, several technical problems and long (21-28 days) assay times. In preliminary studies performed some years ago by us, in less than 25% of SCLC specimens could even 3 drugs be tested at a single concentration. More recent data from our Branch indicates that only 35-40% of recently established SCLC and NSCLC lines can be utilized in clonogenic assays. As a result, we abandoned clonogenic assays for clinical testing of lung cancers nearly 3 years ago.

For clinical studies we use the Weisenthal dye exclusion assay. The advantages of this assay include (1) ability to test most cell lines and some tumors; (2) rapid assay time (4 days); (3) visual separation of tumor from stromal cells; and (4) lack of necessity for a strict single cell suspension. While our data indicate correlation with clinical responses (see below), the Weisenthal assay has major drawbacks that prevent us from using it as a clinical tool and have induced us to explore an alternative testing method. These disadvantages include (1) cell clumping. The requirement of considerable professional and technical time in a extremely tedious assay.

While initially using the Weisenthal assay for clinical testing we have had extensive experience with the semi-automated MTT tetrazolium dye assay, originally developed by Mossman, but considerably modified by us in collaboration with Dr. Jim Mitchell of the Radiation Oncology Branch. While this assay has been used for many biological studies, we are just now preparing to use it for our clinical protocol. Its advantages include (1) rapid (4 days); (2) objective; (3) semi-automated; (4) highly reproducible; (5) some clumping is tolerated. It can generate data much faster than they can be analyzed. Its major disadvantage is its ability to separate malignant from stromal cells.

Major Findings:

The Changing Histology of Lung Cancer

During the past decade occasional reports have documented a major evolution in the histopathology of lung cancer. We cannot estimate the NSCLC:SCLC ratio as SCLC patients are frequently referred to us from outside institutions. Squamous cell carcinoma, the dominant form throughout the world, is experiencing an absolute and relative decline in incidence in this country, accompanied by an absolute and relative increase in the incidence of adenocarcinoma. The 3:1 ratio of NSCLC:SCLC has remained unchanged. We have noted the same trend in NSCLC (1). An analysis of 98 NSCLC protocol patients seen during the past 3 years indicates that the trend is escalating. The histopathology is as follows: Adenocarcinoma 63%, squamous cell 13%, large cell 17%, adenosquamous 2%, other 5%. Peripheral bronchioloalveolar carcinomas are being diagnosed with increasing frequency. Some of these changes, but by no means all, may be attributed to changing diagnostic criteria. We have used the relatively simple criteria formulated by the World Health Organization (WHO). However, we believe that research institutions should apply more sophisticated diagnostic techniques for greater precision.

Cell culture of lung cancers

SCLC.

SCLC specimens are obtained from routine staging procedures and occasional elective sampling of easily accessible metastases as stated in the Extensive Stage Protocol. These studies are very labor intensive as tumor cells are

present only in a mean of 1: 2.7 specimens. Numbers of viable tumor cells frequently are low, and in vitro amplification is almost always necessary. Specimens are cultured in HITES medium, with and without 2% bovine serum or other growth factors. In 46% of protocol patients sufficient amplification has been achieved to permit in vitro drug testing. Most of these become continuous cell lines. Corresponding B lymphoblastoid cultures have been established in routine serum containing medium from several patients and serve as controls for various studies. Of about 80 SCLC lines established by this laboratory, approximately 30 have been started from patients on currently active clinical protocols. In prior years most SCLC lines were estabilished from previously treated patients, as these are easier to establish. As a result of the present protocol studies, a large panel of lines have been established from untreated patients, and their chemosensitivity patterns can be compared to the patients' responses. Other biologic studies have demonstrated the relative rarity of the variant subtype and myc gene family amplifications in lines from untreated patients.

NSCLC

The greater heterogenity of NSCLC cell types (1) and our relative inexperience with this tumor type present considerable problems. After several refinements, we have devised a basic medium (ACL-4) for the culture of large cell and adenocarcinomas 2, 3). However, because of heterogeneity of tumor growth factor requirements, we have to use several variations of the basal formula for initial culture. Also, ACL-4 medium will not suport squamous differentiation. While we lack a satisfactory medium that will permit both cell division and a moderate degree of squamous differentiation, we use our modification of a medium devised by Rheinwald for head and neck tumors. However, most well differentiated squamous tumors undergo terminal differentiation and we are forced to use about 5 media variations for initial NSCLC culture. With these techniques, we can amplify about 25% of adequate NSCLC tumor specimens sufficiently for drug testing. Formulae of our media are presented in Table 1.

Despite these problems, we have established nearly 40 NSCLC cultures, representing nearly 50% of those in the literature. In addition, many of our cultures are unique including bronchioloalveolar carcinomas with type II pneumocyte and Clara cell differentiation, mucoepidermoid, carcinoid, adenosquamous and NSCLC-NE lines.

Neuroendocrine differentiation in lung, colorectal and other human tumors

NE cells represent a widely distributed network of endocrine cells whose predominant function is the synthesis, packaging and secretion of peptide and amine hormones. They share many properties with neurons, including L-dopa decarboxylase (DDC, the key enzyme required for biogenic amine synthesis), cytoplasmic dense core granules (DCG, the storage site of the peptide and amine

products), chromogranin A (CgA, the major matrix protein of the granules), neuron specific enolase (NSE, an acidic form of the glycolytic enzyme enolase) and certain surface antigens charcteristic of natural killer (NK) cells. In previously published articles we and others have demonstrated that SCLC tumors and cell lines express all of these properties and thus are typical NE tumors (4-6). In addition, SCLC lines frequently elaborate and secrete several peptides, the 5 most common being BN/GRP, calcitonin, neurotensin, ACTH and arginine vasopressin (7). As BN/GRP and calcitonin are also elaborated by NE cells present in the normal bronchial mucosa, we regard these as examples of eutopic secretion and the other peptides as examples of ectopic secretion.

To determine the incidence of NE differentiation we assayed DDC concentrations in 436 human tumors representing 40 tumor types. Neuroendocrine tumors expressed high incidence (82%) and high levels (mean value of 86 units/mg). They included pituitary adenomas, medullary thyroid carcinomas, SLCL, bronchial and GI carcinoids, islet cell tumors, carotid body tumors, Merkel cell tumors, pheochromocytomas and neuroblastomas. Other endocrine tumors (adrenal cortical, parathyroid, granulosa/theca cell and non-medullary thyroid) lacked expression, as did melanomas, sarcomas, germ cell tumors, leukemias, lymphomas, mesotheliomas, thymomas, glioblastomas and meningiomas. However, 20% of non-endocrine carcinomas expressed modest levels (12 units). Most of these tumors were NSCLC and colorectal (12 and 51% positive respectively).

Because of these findings, we studied concordant expression of multiple NE markers in NSCLC and colorectal tumors and lines in collaboration with Mark Israel and Lee Hellman. The markers included DDC (measured radiometrically), DCG (ultrastructural examination), CgA and NSE (mRNA expression). There was 98% concordance between CgA and DCG (45/46). However all 4 markers were expressed concordantly in 3/8 NSCLC and 1/25 colorectal tumors expressing DDC. We have established 6 NSCLC and 1 colorectal cell lines that express multiple NE markers. Most carcinoid and classic SCLC tumors and lines expressed all NE markers.

We examined DDC expression in NSCLC subtypes. Because data from tumors (129) and cell lines (31) were similar, they were pooled. DDC was expressed by 19/160 (12%) of all NSCLC, 12/78 (15%) of adenocarcinomas, 7/35 (20%) of large cell carcinomas, 0/32 squamous cell carcincomas, and 0/15 other types. Thus NE expression appears limited to adenocarcinomas and large cell carcinomas. As discussed in another section, the drug sensitivity patterns of 4/5 NSCLC lines expressing multiple NE markers were similar to those of SCLC lines and more sensitive than those of other NSCLC lines.

Variant SCLC lines have altered morphology and growth properties, and selective loss of NE properties (8,9). They are frequently associated with amplification and over expression of the c-myc gene. Most arise as a result of tumor progression events as the variant phenotype is rare in SCLC at presentation (6%).

Expression of natural killer cell antigens by neuroendocrine tumors

Leu-7, a cell surface antigen associated with NK cells, is expressed by neural and NE cells and tumors including SCLC (6). We investigated expression of 4 distinct NK associated antigens on lung cancer cell lines using flow cytometry and immunohistochemistry. The antigens were expressed discordantly - NKH-2 and Leu-11 were either not expressed or expressed at very low levels by all types of lung cancer. By flow cytometry, NKH-1 was expressed by 20/21 and Leu-7 by 13/21 cell lines expressing NE features (classic and variant SCLC, carcinoids, NSCLC-NE). NKH-1 was not expressed on any of 13 other NSCLC lines while Leu-7 was expressed by 2/13. There was excellent concordance between flow cytometry and immunohistochemistry. While Leu-7 antigenicity survived formalin fixation, NKH-1 antigenicity was destroyed. Thus, while NKH-1 is a consistent marker for all types of NE lung tumors, it cannot be applied to routine paraffin sections. The functions of these antigens on NK and NE cells are not known.

Chromosome 3p abnormalities in lung cancer cell lines

Previously we demonstrated a deletion 3p (14-23) in SCLC lines (10). The incidence of deletion 3p in SCLC has been disputed with the Dartmouth group reporting it in only about 20% of their SCLC lines. However, recent data from alternative techniques including restriction length fragment polymorphism (discussed elsewhere) support the concept that del 3p is frequent if not universal in SCLC tumors and lines. In addition we have collaborated with Dr. York Miller (Denver VA Hospital) to measure amino acylase-1 levels in lung cancer lines. The gene for this enzyme is located at 3p(21). While non-SCLC lines expressed levels similar to other human tumors and lines, about one third of SCLC lines express normal levels, one third low levels and one third undetectable levels. As only one intact gene appears necessary for expression, these data confirm and extend the cytogenetic studies. They suggest that submicroscopic deletions may involve the apparently normal chromosome 3 in at least a subset of SCLC cases.

In collaboration with Jacqueline Whang-Peng, we have investigated the incidence of 3p abnormalities in all forms of lung cancer. The abnormalities are multiple in type, and include the usual interstitial deletion as well as terminal or complete deletions of 3p, inversion 3p, isochromosome 3q, translocations involving several other chromosomes, and complete absence of chromosome 3. In all cases there are break points or loss of genetic material from the region 3p (14-23). Ninety-four percent of SCLC lines and tumors (26/28 classic, 5/5 variant) had abnormalities of 3p. Of considerable interest, all four carcinoid line (1 typical and 3 atypical) had 3p abnormalities. Of other lung cancers the abnormality was present in 25% (0/4 NSCLC-NE, 4/17 other NSCLC, 2/3 mesotheliomas).

Thus tumors arising from cells committed toward NE differentiation (SCLC and carcinoids) are frequently associated with abnormalities of 3p. Lung cancer arising from cells committed to other forms of differentiation (NSCLC) or from cells expressing biphasic differentiation NSCLC-NE) have a lower frequency of 3p abnormalities.

Characteristics of NSCLC cell lines

The wide variety of NSCLC lines is evidenced by the multiple phenotypes: poorly and well differentiated adenocarcinomas, bronchioloalveoar carcinomas (Clara cell, mucin secreting and type II pneumocyte varieties), poorly and moderately differentiated squamous cell, adenosquamous, mucoepidermoid and large cell. NSCLC lines expressing NE features have already been described.

We compared expression of markers for squamous differentiation in NSCLC lines having morphologic expression of squamous differentiation (n=5) with other NSCLC (n=10) and SCLC lines (n=10). Squamous lines expressed the following markers: EGF receptors, transglutaminase activity, involucrin, crosslinked envelope formation and medium molecular weight keratins. Other lines lacked these markers or expressed them inconsistently and at lower levels (11).

In collaboration with Jeff Whitsett (University of Cincinnati), we studied expression of surfactant associated protein, mol. wt. 35,000 (SAP 35) in 44 lung cancer lines. Five bronchioloalveolar carcinoma lines of diverse subtypes expressed moderate to high levels; other lines were negative. These are the first known observations of SAP 35 expression in human lines. Expression has been demonstrated at protein level (Elisa tests, immunohistochemistry) and at mRNA level (Northern blots). Expression and secretion can be enhanced by dexamethasone.

Culture and characterization of colorectal cell lines

We have established and characterized 14 human colorectal carcinoma cell lines from primary and metastatic sites (12). Nine lines were established in serum supplemented medium, and 5 in ACL-4 defined medium. After establishment, the lines could be grown interchangeably in either medium. Twelve were tumorigenic in athymic nude mice when injected subcutaneously, and two grew intraperitoneally as well. Based on culture, xenograft and ultrastructural morphology, the 14 lines coud be subtyped as follows: well differentiated (n=4); moderately differentiated (n=5); poorly differentiated (n=4); mucinous carcinoma (n=1). Membrane associated antigens) were expressed by 50 - 85% of the lines, although there was no correlation with degree of differentiation. Lines expressing CEA and CA19-9 actively secreted these antigens, while TAG-72 was not secreted.

Most lines (13/14) expressed DDC, the enzyme characteristic of NE differentiation. One of these lines expressed all of the markers of NE differentiation including dense core granules, while other lines expressed NE markers (other than DDC) inconsistently. Of 11 lines examined cytogenetically, 9 contained double minute chromosomes. Three of these lines also contained homogeneously staining regions. These findings indicate a high incidence of amplification of one or more as yet unidentified genes. Amplification of c-myc gene was present in one line, while all lines expressed c-myc mRNA. Thus c-myc amplification does not account for the gene or genes amplified in the majority of our colorectal lines.

Clinical Testing of Lung Cancers

The Weisenthal assay has been used exclusively for clinical testing of specimens from patients on protocol studies. Tumor cells are partially disaggreated and incubated with drug concentrations for 1 hour or continuously. After 4 days nucleated duck red cells are added as an internal control and also fast green/nigrosin dyes. The dyes stain dead cells but are excluded from living cells. Cytospin preparations are counterstained. The ratio of living tumor cells to duck cells determined and expressed as a percentage of the control sample ratio. Tumor cells are separated from stromal cells by cytological criteria. While 3 drug concentrations are tested, a single reference concentration is used for clinical decisions. A drug is considered active if it results in more 50% kill at the reference concentration. The reference concentrations were selected by Dr. Larry Weisenthal after extensive testing of many lung cancer tumors. However, after further testing by both our groups, the concentrations and exposure times have been altered for cisplatin and methotrexate.

While testing of fresh specimens is performed when clinically indicated, it is not performed electively. The cell numbers of tumor specimens drop during the 4 day incubation while cell lines increase in number. The resultant dead cells cause technical difficulties and wide variations between replicates. For these resons we usually culture tumor samples for varying periods prior to testing. Testing is performed whenever sufficient amplification of tumor cell number has occured. Stomal cell numbers are suppressed by (1) use of selective media (see previous section) and (2) differential substrate attachment. Cell lines also permit repeat testing and relatively low assay variability.

SCLC lines are tested against 7 drugs of known clinical activity in this disease. Of 46 patients from whom 1 or more tumor containing specimens reached the laboratory, testing was performed on 21 (46%). The data indicate considerable heterogeneity between lines and between drugs. VP-16 was the most effective, both as an 'active' agent and as 1 of the 3 most effective agents irrespective of 'activity'. Methotrexate was the least effective. As reported elsewhere, there was considerable correlation between the clinical responses of patients to initial therapy (VP-16 and cisplatin) and whether their coresponding lines were active to 2 or more of the 7 agents tested. In addition, patients given a second chemotherapy program based on in vitro testing had higher response rates (25% vs 7%) than those given 'standard' second line therapy (VAC). These data provide justification of the use of tumor cell lines for continued clinical testing as well as for screening new chemotherapeutic agents.

We have tested 21 untreated NSCLC lines by the Weisenthal assay, testing 13 drugs used in previously reported chemotherapy regimens. Of a total of 135 individual drug tests, 54 (24%) were active. These figures are modestly different than for SCLC. Of 21 untreated NSCLC lines tested, 48/135 (36%) of drugs assays were active.

Development of the MTT assay

Because of the major technical problems with the Weisenthal assay, we have attempted to replace it with the semi-automated MTT assay. In this assay, cells are seeded into 96 well plates, drugs added and incubated for 4 days. The tetrazolium salt MTT is then added to the wells. In the presence of the mitochondrial enzyme succinate dehydrogenase MTT is reduced to violetblue colored formazan crystals. Some of the media is removed and replaced with DMSO, which disolves the crystals. The absorbance of each well is measured at 540 nm in an automatic plate reader coupled to a computer which stores the data and does calculations. The data are plotted and the concentration of drug (IC_{50}) required to reduce the cell number number by 50% calculated. Production of the blue formazan product is linear to the cell number over a wide range. After suitable modification, Carmichael and Mitchell, in collaboration with us demonstrated that the MTT assay gave results similar to the clonogenic and dye exclusion assays, and was suitable for the assessment of chemosenitivity and radiosensitivity (14,15).

We have modified the assay further. We have used continuous drug incubation, as this removes the lengthy technical procedure of washing after 1 hr. incubation. In addition, continuous exprosure magnifies minor differences between relatively resistant lines. We have chosen a 4 day incubation period (7 day periods are used for assessment of growth factors). Because the optimum seeding density for each cell line must be determined prior to drug testing, clinical specimens and new lines can be tested over a 8-9 day period. We have chosen to test lung cancer lines in defined medium (HITES for SCLC, ACL-4 for NSCLC) plus 0·2% albumin. In their appropriate defined media lung lines grow as or more efficiently than in serum supplemented media. Use of the defined medium permits the more accurate assessment of methotrexate and 5Fu. Methotrexate in serum containing media, frequently fails to kill 50% of the cells, irrespective of the drug concentration, making IC_{50} determination impossible. Data from other drugs appear very similar in serum free and serum supplemented media. An initial problem was the variability of IC_{50} values between various assays, even through replicate wells within a single assay varied less than 15%. However rank order analysis for each drug as determined in separate experiments showed a highly significant correlation between experiments (16). Since then we have replaced mechanical dispersion of cell aggregrates with gentle trypsinization. The cells are seeded and permitted to recover for several hours prior to the addition of drugs. This technique has greatly diminished inter-test variability.

Chemosensitivity of human carcinoma lines

Thirty human lung cancer lines were tested by the MTT assay, using 7 drugs (16). Cell lines from untreated SCLC patients were more sensitive to adriamycin, melphalan, vincristine and VP-16 compared to NSCLC lines. No significant differences were observed with BCNU, cisplatin and vinblastine. Interestingly, tumor lines from patients receiving prior chemotherapy were relatively resistant to most drugs.

The chemosensitivity of 11 human colorectal lines to the same 7 agents was also determined (17). From the data, we predict that 5-FU is the sole active agent tested. This data is based on two observations: the range of IC_{50} drug concentrations was greatest with 5-FU (388-fold compared to 5-30 fold for the other agents); and the area under the curve which produced 50% inhibition was within a clinically achievable range only for 5-FU. However, even 5-FU appeared active against only 2 of the 11 lines. Of major interest, for all 7 agents tested the mean IC_{50} concentrations were in the rank order (of increasing resistance): SCLC untreated; SCLC treated; NSLCL; colorectal (16,17,18). These findings, of course reflect clinical experience.

As previously mentioned, about 12% of NSCLC tumors express neuroen docrine (NE) properties. We have established 6 NSCLC lines expressing multiple NE properties (NSCLC-NE). We compared the in vitro drug sensitivity patterns of NSCLC-NE lines (n=5) with those of other NSCLC (n=12) and SCLC (n=10). All lines were from previously untreated patients. Five drugs were tested. Four of the NSCLC-NE lines were highly sensitive - among the 6 most sensitive of all NSCLC lines to all 5 drugs. However the fifth NSCLC-NE line was relatively resistant. When compared by the Mann-Whitney U rank order test SCLC lines were significantly more sensitive than the NSCLC lines, but were indistiguishable from the NSCLC-NE lines. However, for 3 drugs NSCLC-NE lines were more sensitive than other NSCLC lines. Of interest, one of the untreated SCLC lines was also highly resistant. While we have established and tested only a small number of NSCLC-NE lines, their chemosensitivity patterns appear to more closely resemble those of untreated SCLC lines than of other NSCLC lines. The incidence of NSCLC in this country is about 109,000 cases annually, and an estimated 13,000 of these will express multiple NE features. Thus, an important subpopulation of NSCLC tumors may prove to be relatively responsive to chemotherapy. This concept is currently being tested in a prospective clinical trial.

Synergism between VP-16 and cisplatin

VP-16 is one of the most active single agents in SCLC, while cisplatin is considerably less active as a single agent. In vitro testing confirms these patterns - VP-16 is the most active of the 7 agents tested, while cisplatin is the 4th most active. Combination studies using in vivo animal models suggested that the 2 drugs may act synergistically. Little or no human cell line data exist. Despite the relatively scant animal data, VP-16 and cisplatin are one of the commenest drug combinations used in the therapy of lung cancer. In fact, this combination represents the front line therapy utilized in our clinical protocols for both SCLC and NSCLC.

We tested the effects of VP-16 and cisplatin combinations on SCLC lines sensitive and resistant to these agents. Data were analyzed by isobolograms in which each point represented combinations resulting in 50% cell kill (ie

one IC_{50} unit). The combinations were tested in checker board fashion, a complete range of drug B concentrations run with each concentration of drug A and visa versa. For each line approximately 50 combinations and controls were tested. Because initial testing of a sensitive cell line indicated antagonism, lines were tested in serum free and serum containing media.

Of interest in serum containing media the agents acted synergistically, but in serum free media the effects were more modest. While we do not know the reasons for these different effects, we presume that the data from serum containing media represent the interactions occuring in vivo.

Proposed Course

Biology and growth of human cancers. We wish to determine more precisely the growth factor requirements of lung and colorectal tumors. We intend to test the components of ACL-4 medium singly and in combination. We will also test there requirements for maintaining or inducing squamous differentiation. By these methods we hope to devise media more useful for the culture of the various differentiated forms of lung cancer.

While we are using several markers to detect NE differentiation in NSCLC, we do not know the most sensitive methods to detect NE markers or the significance of expression of a single marker. We are currently testing a comprehensive marker panel (DCG, DDC, CgA, NSE, CK-BB, BN/GRP, calcitonin) in NSCLC lines and tumors. We will compare the sensitivity of several methods (enzymatic, mRNA expression, immunologic, immunohistochemical). This will provide a large data base for biological and clinical correlations. We will explore whether some of the tumor markers may have clinical applications. BN/GRP is useful for detecting CNS metastases of SCLC (12). Currently, chromogranin A, which is stable, actively secreted and present in all NE tumors (except variant SCLC) appears promising.

We have obtained mono- and polyclonal antibodies to SAP 35 and other proteins associated with surfactant. They will be used to screen our cell lines for expression ion and the data will be correlated with ELISA assay results. In addition, we will collect a large number of human lung tumors in collaboration with Dr. Bruce Mackay (Houston) who will examine them ultrastructurally. They will also be screened for SAP 35 expression. These studies will help confirm our pathological findings that the incidence of bronchioloalveolar carcinomas is increasing rapidly. Some of these tumors will provide a source of mRNA for a variety of studies including SAP 35 and chromogranin expression (suitable probes already exist). Expression of the neu proto-oncogene is associated with peripheral airway cells. We will explore its expression in bronchioloalveolar carcinomas.

In vitro drug testing. The most important objective is to apply the MTT assay for testing of clinical specimens. While all continuous cell lines can be tested, we are trying to determine how soon after arrival various types of tumor containing specimens can be tested, and the optimal methods of obtaining pure or almost pure tumor cell populations. The use of defined media greatly assist this process. Identification of active agents and their rank order is more complex. We intend to complete testing of all SCLC cell lines from protocol patients (approx imately 30 lines) as well as every NSCLC line established in this laboratory (approximately 35 lines). Small cell lines will be tested with 7 and NSCLC lines with about 10 agents. All lines are tested at least twice, and if the data are discordant three or more times. This will generate a large, comprehensive data base which will permit correlation with clinical responses with the SCLC patients and some of the NSCLC patients. Comparisons between in vitro areas under the curve and those achievable in vivo will also be explored.

Comparisons between the chemosensitivity of tumor cells and corresponding autologous cells will be performed. Many paired B lymphoblastoid - SCLC lines exist. The feasability and relevance of using marrow cells in the MTT assay will be studied. Initially we will test mouse marrow cells using drugs for which mouse marrow toxicity data exists. If the MTT data correlates with the in vivo data, we will test human marrow samples obtained during routine staging procedures. Mesothelial, filroblast and other stromal cells are also potential sources. Depending on the amount of variation of normal cell sensitivity encountered between individuals, they may serve either as individual or generalized reference points.

The MTT assay will be used to test new agents of outstanding general interest, those targeted against specific tumor types as well as all agents being considered for phase I-II studies in this Branch. Other miscellaneous projects include extending VP-16 and cisplatin combination studies to NSCLC lines, testing other presumably synergistic combinations and determining the optimal agents used for routine clinical testing. For instance, we currently use nitrogen mustard as a substitute for cytoxan which is inactive in routine in vitro assays. Other possible substitutes such as melphalan will be tested. CCNU presents almost insurmountable solubility problems. BCNU, which is much more soluble will be substituted if it gives comparable data.

Clara cells are metabolically very active and express high amounts of cytochrome P-450 oxidase activity including certain specific isoenzymes. Clara cells may be selectively destroyed by 4 - ipomeanol which is transformed into a highly reactive metabolite in Clara cells and type II pneumocytes to a lesser extent. This agent is about to enter phase I studies in NSCLC. We will test several lung cancer cell line for relative sensitivity to 4 - ipomeanol, and correlate results with expression of SAP 35 protein.

Finally, we plan to validate our <u>in vitro</u> chemosensitivity results for the colorectal cell lines in a nude mouse xenograft model. This is particularly important since the present NCI disease-targeted drug screening program rests upon <u>in vitro</u> testing of human-derived cell lines.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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PERIOD COVERED

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Second Messenger and Receptor Systems in Human SCLC

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

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Molecular Genetics Section

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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.)*

Previous studies with small cell lung cancer (SCLC) cell lines have demonstrated that gastrin-releasing peptide (GRP), the mammalian counterpart to bombesin, is expressed in a significant fraction of SCLC lines. To assess its role in causing autocrine stimulation, initial experiments attempted to demonstrate specific binding to SCLC membrane fractions, with little success. Accordingly, efforts to define rapid physiologic responses to GRP were undertaken. These studies demonstrated clearly that in 5/11 SCLC cell lines tested there was evidence of calcium mobilization following GRP or bombesin stimulation. The structure-activity relationship of this effect was in accord with that expected for GRP receptors in gut, brain, and anterior pituitary cells. Further studies focused on the relationship of this response to inositol phosphate metabolism. In a SCLC cell line with a brisk response to GRP with increased intracellular calcium, there was evidence of increased inositol 1,4,5 trisphosphate within seconds of addition of bombesin congeners. Both the mobilization of calcium and inositol phosphate turnover were inhibited by cholera toxin and active phorbol esters In the presence of pertussis toxin there was also a less complete stimulation of inositol phosphate turnover. In corollary studies it was demonstrated that the cell lines with the best response to bombesin showed constitutive expression of L-myc and prepro GRP without expression of c- or N-myc. In constrast, cell lines without evidence of response to bombesin had constitutive N- or c-myc expression.

These studies suggest that an order of progressively malignant and distinct phenotypes can be defined in SCLC cell lines. A most "differentiated" cell line would be those which produce and respond to GRP, and also produce L-myc. A less differentiated cell line would neither produce nor respond to GRP and express abundant c- or N-myc. GRP (+), L-myc (+), c-myc (-), and N-myc (-) cell lines would represent those in which an effort to block a potential autocrine loop involving GRP might be most successful.

GPO 914-918

PROJECT DESCRIPTION

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	Leonard Neckers, Ph.D.	Senior Investigator	LP-NCI
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Objectives:

1. To examine the mechanism of bombesin induced calcium fluxes in responding small cell lung cancer cell lines, and discern the differences in calcium storage and release between bombesin-responsive and bombesin non-responsive cell lines.

2. To define mechanisms of bombesin-induced phosphatidylinositol turnover in responding lung cancer cell lines.

3. To understand the differences between bombesin - responding and bombesin non-responding small cell lung cancer cell lines with respect to inositol lipid and inositol phosphate metabolism.

4. To examine the participate of guanine-nucleotide binding proteins in bombesin-induced signal transduction in lung cancer.

5. To characterize the bombesin-associated tyrosine kinase in small cell lung cancer cell lines, and place this in the context of other tyrosine kinase genes known which may be expressed in small cell lung cancer cells.

6. To d termine if bombesin action results in altered protein kinase C activity or distribution in small cell lung cancer cell lines.

7. To determine if bombesin responsive and bombesin non-responsive small cell lines have constitutively altered protein kinase C activation, distribution, or substrates.

Methods Employed

Calcium metabolism: Quin-2 spectrofluorometry.

Inositol Turnover: Metabolic labelling with radioisotype; high performance liquid chromatography; thin-layer chromatography.

Tyrosine kinases: Western blotting; anti phosphotyrosine antibodies; oligonucleotide synthesis and hybridization.

Guanine nucleotide binding protein: ADP-ribosylation of membrane fractions; Northern blotting with DNA-RNA hybridization; cDNA library construction.

Protein kinase C: Assays of kinase.

Major Findings

Initial experiments have sought to define the tvpe of calcium mobilization observed in response to bombesin in small cell lung cancer, and to correlate this with the biologic features of the available cell lines.

We have observed that bombesin congeners mobilize Ca^{2+} from intracellular stores as assayed by Quin2 in some (5/11 tested) small cell lung cancer cell lines. Not all of the Ca^{2+} appears to arise from intracellular sources, as EGTA in the external medium attenuates but does not eliminate the release of calcium. The calcium response, once elicited, causes the cells to be refractory to further stimulation by bombesin unless the ligand is removed. Of interest, the cell lines with the best Ca^{2+} response to bombesin have no evidence of constitutive c- or N-myc expression, and all responders thus far assayed express abundant GRP and easily detectable L-myc transcripts. Cell lines without evidence of a Ca^{2+}-transient response to bombesin do not show GRP transcription, and have abundant c- or N-myc expression with variably occurring L-myc transcripts. Thus, these data would suggest that constitutive expression of c- or N-myc removes a potential need for bombesin related Ca^{2+} mobilization in promoting cell growth. It should be underscored that a major unanswered question is whether the c- or N-myc expression causes independence from bombesin induced stimulation or whether constitutive c- or N-myc expression reflects activation of a process usually under hormonal influence in those cells without constitutive c- or N-myc expression. The importance of this question lies in the possible pharmacologic manipulations of a system whose deregulation leads to hormone independence with c- or N-myc expression.

In this regard, it is of interest that preliminary experiments have suggested that active phorbol ester treatment attenuates the Ca^{2+} mobilization observed in response to bombesin in responding cell lines; that diltiazem blocks a fraction of the observed rise in intracellular Ca^{2+} in response to bombesin; and that cholera toxin (with greater efficiency than pertussin toxin) likewise blocks the increase in intracellular Ca^{2+} in response to bombesin.

Preliminary experiments have also demonstrated that a cell line with a Ca^{2+} response to bombesin does possess a bombesin-responsive tyrosine kinase activity, as assayed by use of an anti-phosphotyrosine antibody. There is an appreciable background activity with the same species or substrate. Likewise, in a further characterization of tyrosine kinase genes active in lung cancer cell lines, using cDNA clones derived to the LSTRA (lck) gene, 9/10 small cell lines but 1/10 non-small cell lung cancer cell lines have evident lck expression. Whether this is related to the expression of the bombesin related tyrosine kinase is unclear. Whether other tyrosine kinase genes may be found expressed is also unknown.

In initial experiments, we have defined that bombesin congeners do activate phosphatidylinositol turnover in a cell line shown previously to produce gastrin-releasing peptide, the mammalian homolog of bombesin. This response is initially seen at doses as low as 1 nM, and increases until a maximal extent of turnover occurs between 100 and 1000 nM. The response is observable as rapidly as measurements can be made after Tyr-4-bombesin addition to intact cells, with initial production of inositol 1,4,5 trisphosphate, and decline in this isomer over 30-45 seconds and appearance of inositol 1,3,4 trisphosphate. The latter compound is the product of inositol-5-phosphate phosphomonoesterase action on inositol 1,3,4,5 tetrakis phosphate. The latter compound appears to be present at relatively high levels (by label incorporation) in comparison to the inositol 1,4,5 trisphosphate isomer. The methods to complete these studies have employed two high performance liquid chromatography systems which can resolve physiologically relevant IP1, IP2, and IP3 isomers as well as IP4. These studies have also established that bombesin stimulation of responding cell lines is not accompanied by detectable gross changes of ATP, GTP, or other potentially relevant nucleotide levels.

The structure-activity analysis completed thus far suggests that GRP and GRP (20-27) are active in stimulating the appearance of inositol metabolites, both in short term (<40 sec) experiments and in the tonic elevation in the presence of Li^+ of inositol -1- and -4- phosphates over 20-30 min of incubation with congeners. Preliminary experiments suggest an effect of active phorbol esters to diminish the appearance of these latter compounds. A cell line not containing GRP mRNA, and with low to no detectable bombesin immunoreactivity has no evidence of responsiveness to bombesin congeners to increase PI turnover. Deacylation of component membrane lipids does not suggest absence or unavailability of lipid substrates to explain this.

Preliminary experiments have likewise demonstrated that membrane fractions from cells known to respond to bombesin congeners have both cholera and pertussis toxin substrates, and pretreatment with cholera and pertussis toxin in vivo does effect alteration of these toxin substrates so that they are less available to in vitro modification after membrane isolation.

Thin layer chromatographic TLC (systems) to separate the major phospholipid metabolities, notably phophatidic acid, PI, PIP, PIP2, have been developed. In addition, other systems to examine diacylglycerol levels by TLC have also been set up. Preliminary experiments have established labelling conditions and extraction techniques to examine these metabolites.

Significance to Biomedical Research and the Program of the Institute

These studies are focused at two overall types of goals. First, a definition of the pathways activated in small cell lung cancer cell lines by bombesin, and second a definition of the different phenotypes of small cell lung cancer with respect to response to bombesin. These issues are of importance because successful design of treatment strategies may use this information to assist in the planning, for example, of bombesin analogs, or of agonists that might function beyond the level of the cell surface receptor. Second, it may be possible, using this information, to perform diagnostic tests on tumor derived material to

discern which patient's tumors may best benefit from application of approaches to interrupt autocrine activation of the tumor cells.

Proposed Course

Our efforts will include the completion of ongoing studies describing the activation by bombesin congeners of calcium fluxes and inositol turnover in SCLC. This will include detail description of the modulation of this response by protein kinase C activators, cholera toxin, pertussis toxin, and cyclic nucleotide modulators. We will characterize the expression of guanine nucleotide binding protein genes using heterologous crossed species probes available to us. We will assess evidence of endogenous turnover of inositol phosphates potentially uncoupled from receptor activation in those cell lines not responsive to bombesin or assessed by transient calcium responses. Further efforts to identify the receptor complex for GRP using cross-linking agents will continue. Finally, as we have evidence of increased phophotyrosine in a 115 Kd species after bombesin stimulation, we will attempt to screen an appropriate small cell lung cancer cDNA library with a consensus tyrosine kinase oligonucleotide probe in an attempt to find the bombesin sensitive kinase cDNA and directly assess its expression in small cell lung cancer.

Publications

Trepel, J.B., Moyer, J.D., Heikkilla, R., Neckers, L.M., and Sausville, E.A.: Relationship of bombesin responsiveness to myc family gene expression in small cell lung cancer. Clinical Res. 35: 528A, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06592-02 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Lineage-Specific Marker and Proto-oncogene Expression in Human Lung Cancer

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

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	Adi Gazdar, M.D.	Senior Investigator	NCI-NMOB
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	Mark Levitt, M.D.	Clinical Associate	NCI-NMOB
	Michael Birrer, M.D.	Clinical Associate	NCI-NMOB
	O.W. McBride, M.D.	Senior Investigator	NCI-DCBD

COOPERATING UNITS *(if any)*

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Molecular Genetics Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	0	0

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☐ (a1) Minors

☐ (a2) Interviews

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

Summary of Work

A) CPK-BB - Creatine phosphokinase BB isoenzyme is present in small cell lung cancer as compared to other histologic types. Using a CPK-B clone derived in rabbits, human cDNA clones were generated from a SCLC cDNA library. Use of the isozyme-specific probe confirmed increased expression of CPK-BB at the transcriptional level in small cell as opposed to non-small cell lung cancer. Using the isozyme specific probe, CPK-B was unexpectedly shown to have two different chromosomoal locations in humans, suggesting two CPK-B genes. A genomic library from human placenta has yielded genomic clones whose structure is being defined. Definition of the promoter region will allow examination of the factor(s) present in neuroendocrine tissue which mediate the abundant expression of this gene.

B) Neuron-Specific Enolase - Further characterization of the clones derived during the past year suggests at least three types of enolase-related genes have been cloned. The precise structure of these genes is being pursued.

C) EGF-Receptor Gene Expression - Having defined in the past which non-small cell lung cancer cell lines express the EGF receptor, efforts are now focusing on evidence for receptor function. Experiments suggest that there is evidence of EGF-receptor kinase stimulation by EGF and TGF- . Further studies are to focus on whether this response can be specifically blocked by antibodies to the EGF receptor.

D) Ras Gene Expression - Efforts were made to correlate the expression of ras genes with the response of some small cell cancer lines to release Ca^{2+}. Further work will focus on whether the ras genes present in these cell lines are germ line or mutated at positions known to promote tumorigenesis.

PROJECT DESCRIPTION

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Professional Staff:

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Objectives:

1. To determine the basis for the selective expression of genes related to the neuroendocrine phenotype in small cell lung cancer lines.

2. To examine the function and expression of the EGF receptor gene in non-small cell lung cancer cell lines.

3. To assess the relationship of *ras* gene expression to biologic aspects of tumor cell metabolism in small cell lung cancer cell lines.

Methods:

1. Recombinant DNA techniques: cDNA library construction, subcloning, DNA sequencing of derived clones, oligonucleotide synthesis.

2. Cell biologic techniques: cell culture, *in vitro* metabolic labelling of permeabilized cells.

3. Biochemical techniques: assay of Ca^{2+} by quin-2 technique; DNA/RNA hybridization.

Major Findings:

We have used cDNA probes derived from non-human species to isolate and characterize cDNA clones for the human creatine phosphokinase B subunit, and putative clones for neuron-specific enolase. The creatine phosphokinase B clones were characterized in greatest detail during the past year. Significant findings to emerge from these studies included the definition by sequence analysis of a portion of the CPK-B cDNA which is B isoenyzme specific. This fragment allowed us to establish that the high enzymatic level of CPK-BB in human small cell lung cancer reflected greatly increased steady-state transcript levels in comparison to non-small cell lung cancer cell lines thus far tested. Furthermore, this probe showed that the human CPK-B cDNA has two chromosomal locations, one on chromosome

14, the other on chromosome 16. This implies either the existence of a second, CPK-related gene or pseudogene. Moreover, the probe demonstrated the existence of two EcoRI polymorphisms independently involving the CPK gene(s). Further definition of these findings will require an understanding of the genomic DNA representation of the CPK-B gene(s).

The neuron specific enolase related clones that were defined previously have been further characterized by restriction mapping and partial sequence analysis. One clone clearly has homology expected for a neuron-specific isoenzyme. The other two classes of clones are related but not identical. Complete sequence analysis will be of importance in establishing the relationship of these species.

The definition of EGF-receptor-positive non-small cell lung cancer cell lines by I^{125}-EGF binding and expression of appropriate transcripts allowed further studies this year of the biochemical activation of these species in an adeno-squamous cell line. Stimulation of thymidine incorporation and stimulation of the EGF-receptor kinase was observed. These results suggest that in human lung cancer the EGF receptor is not simply a marker for the epidermoid phenotype, but also can be functional as well to promote growth. In addition, TGF- was shown to cause analogous effects, and therefore be of potential clinical importance in directing tumor cell growth.

Ras genes have been implicated as mediating biochemical signal transduction in an aberrant fashion in tumors. Previous studies have defined that ras gene expression is frequent in human lung cancer. As N-ras is suggested to be coupled to the bombesin induced response in model systems, efforts to define N-ras relations to bombesin stimulation demonstrated that at the level of Northern blot analysis no strict correlation between bombesin responsiveness and N-ras expression was observed.

Significance to Biomedical Research/Institute Program

These studies will allow definition of elements responsible for maintaining the unique phenotypes present in the diverse histologic types of human lung cancer. Such elements could consist of intracellular control mechanisms to allow augmented transcription of lineage specific markers (CPK-BB; NSE); such elements could consist of coupling mechanisms of a growth factor receptor to events proceeding intracellularly (potentially N-ras); finally, another element could be to affect receptors of one type (EGF receptor) by endogenously produced stimulatory ligands (TGF's). Each of these steps represents a potential point where therapeutic agents might be developed or directed.

Proposed Course

The studies to be continued here represent an intensified focus on the small cell elements. The EGF-receptor related aspect will be terminated after effects of antibodies to the EGF receptor are explored, unless striking effects of inhibition of growth by the antibodies are demonstrated.

The characterization of the CPK-B genomic structure (derived from chromosome 14) will be completed, and efforts to define transactivating factors in extracts of small cell lung cancer cells and to establish the activity of the promoter in heterologous environments will be undertaken.

The characterization of the derived enolase cDNA clones will be completed, and genomic clones for each of these identified and characterized. Chromosomal localization will be attempted.

Synthetic oligonucleotide specific for position of ras genes frequently observed to be mutated with resultant activation will be used to characterize the frequency of ras mutation in small cell lung cancer. Efforts to correlate this with bombesin-responsiveness will be undertaken, as well as correlation with expression of other oncogenes (notably L-myc).

Publications (During 1986-1987)

Kaye, F.J., McBride, O.W., Battey, J.F., Gazdar, A.F., and Sausville, E.A.: Human creatinse kinase B complementary DNA. Nucleotide sequence, gene expression in lung cancer, and chromosomal assignment to two distinct loci. J. Clin. Invest. 79: 1412-1420, 1987.

Levitt, M.L., Gazdar, A.F., and Sausville, E.A.: Biologic activity of epidermal growth factors in human lung cancer cell lines. Proc. Am. Soc. Clin. Oncol. 6: 181, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06594-02 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Molecular Genetic Events in Lung Cancer

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

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	John D. Minna, M.D.	Chief	NCI-NMOB
	Daniel C. Ihde, M.D.	Senior Investigator	NCI-NMOB
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.4	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.)*

Summary of Work

A. Thirty-four tumor specimens from small cell lung cancer patients were studied for myc family DNA amplification. None of the 11 tumor specimens obtained from untreated patients had DNA amplification of any of myc family. Six of 23 tumor specimens obtained from chemotherapy treated patients had DNA amplification of the myc family (4 N-myc, 2 L-myc)

B. Twenty-six tumor specimens and normal tissues were obtained from different small cell lung cancer patient treated at the NCI-Navy Medical Oncology Branch. DNA was prepared, digested with appropriate restriction enclonucleases, and hybridized to 4 probes which detect restriction fragment length polymorphisms (RFLP) in DNA.

Twenty-five were heterozygous for at least one allele. The DNA from tumors showed deletion of one of the heterozygous alleles in 23 of the 25 patients. One of the 2 exceptions was a patient with extrapulmonary small cell lung cancer. From these RFLP studies we concluded the deletion of 3p alleles is present and common in small cell lung cancer providing additional evidence for deletion of the short arm of chromosme 3 in small cell lung cancer.

PROJECT DESCRIPTION

RELATION OF MOLECULAR GENETIC EVENTS IN LUNG CANCER WITH PATIENTS' CLINICAL PRESENTATION AND COURSE

Professional Staff:

PI:	Bruce E. Johnson, M.D.	Senior Investigator	NCI-NMOB
Others:	Adi F. Gazdar, M.D.	Senior Investigator	NCI-NMOB
	John D. Minna, M.D.	Senior Investigator	NCI-NMOB
	Daniel C. Ihde, M.D.	Senior Investigator	NCI-NMOB
	James Mulshine, M.D.	Senior Investigator	NCI-NMOB

Objectives

1. Determine the myc family DNA amplification pattern in the new, prospectively charactertized lines.
2. Update the information on the clinical association specifically to see if the amplification pattern changes with the new VP/PLAT induction regimen compared to the CMC-VAP regimen.
3. Study the myc family gene expression using an RNase protection assay for all cell lines established in the prospective cell line study to correlate gene expression with the clinical course of the patients.
4. Simultaneously study normal tissue, tumor cell lines, and tumors from the patient from chromosomal deletions using restriction fragment polymorphisms while having a karyotype performed on the cell line to correlate the evidence for chromosomal deletion using restriction fragment polymorphisms and cytogenetic analysis.
5. Establish EBV Transformed lymphocyte cell lines to enable us to study DNA and Karyotypes of more normal tissues of small cell lung cancer patients who have cell lines established. This will provide additional numbers of patients to study the clinical role of 3p deletion in lung.

Methods:

We have completed analyzing the initial 44 tumor cell lines established from small cell lung cancer patients' tumors treated on protocol at the NCI-Navy Medical Oncology Branch and correlated this with the clinical presentation and course of these patients. We initially grew up all 44 cell lines and prepared DNA. The DNA was hybridized to radiolabelled fragments of the myc family of genes, c-myc, N-myc, and L-myc. DNA copy was considered to be amplified if the signal from the myc family gene was 4 fold greater than a single copy control.

Small Cell Lung Cancer Patients Who Have Cell Lines Established at Diagnosis Have a Shortened Survival.

The analysis of the clinical course of the patients from whom cell lines were established revealed that the 19 patients who had cell lines established prior to the initiation of chemotherapy had a markedly shortened survival compared to other large series of small cell lung cancer patients (9). Eighteen of the 19 patients had extensive stage small cell lung cancer (disease spread outside the chest) so we compared these nineteen patients to the other 123 extensive stage patients treated on the same protocols. The median survival of the nineteen patients from whom cell lines were established was 14 weeks compared to 48 weeks in the other extensive stage patients who did not have cell lines established ($p<0.001$). the previously identified prognostic variables identified in small cell ling cancer were evaluated to see if these would account for the difference in the survival. There was no significant difference in age, sex, stage, or presence of liver metastases or brain metastases. This initial observation of shortened survival in patients with tumor cell lines established at diagnosis has been made on the initial cell lines established in the branch and may not continue to be true in the future. Dr. Gazdar and Dr. Ihde are collecting information prospectively to answer this question.

We believe this information has potential important implications for understanding the use of cell lines to study human cancer. These cell lines which represent the initial efforts of the branch in establishing cell lines from human tumors came from patients who had markdly shortened survival compared to other extensive stage small cell lung cancer patients. Therefore, the initial work on cell lines from untreated patients was from patients who had a shortened clinical course. These cell lines were established from patients with a more aggressive biologic course than the routine extensive stage patients. Other insights are available from childhood neuroblastoma patients. In this disease, the patients whose tumor grows in the laboratory lives a shorter period of time than the patients whose tumors do not grow (10). In addition, nearly all neuroblastoma cell lines are amplified for N-*myc* (11). N-*myc* amplification has been associated with advanced stage disease and more rapid tumor progression in childhood neuroblastoma (6,7). Thus, in both these systems of untreated small cell lung cancer and childhood neuroblastoma there is evidence that the cell lines are more likely to be established form patients with amore aggressive biologic course and these coll lines may not be representative of the broad spectrum of tumor biology. We believe this has important implications when adapting the findings of cell culture to the clinical treatment of cancer.

Myc Family DNA Amplification is More Common in Cell Lines Established from Chemotherapy Treated than Untreated Patients, and c-Myc Amplification is Associated with Shortened Survival

The DNA from 13 of the 44 small cell lung cancer cell lines were amplified for one of the *myc* family oncogenes (5 c-*myc*, 4 N-*myc*, and 4 L-*myc*). *myc* family DNA amplification was more common in cell lines established from chemotherapy treated than untreated patients (11/25 versus 2/19 respectively; $p=0.04$). The

survival of patients whose cell lines established after treatment with chemotherapy had c-myc amplification was shorter than other patients whose cell linesdid not (median 33 versus 53 weeks; p=0.04). the survival of chemotherapy treated patients whose cell lines had N-myc or L-myc amplification was not markedly different than the patients whose cell lines were not.

myc Family DNA Amplification in Tumors is the same as in the Cell Lines Established from the same Patients

We have collected 35 tumors from small cell unf cancer patients adequate for molecular genetic analysis, 6 fromsurgical biopsies and 29 from postmorten examinations. Thirty-four have been analyzed for myc family DNA amplification. Twenty-three tumors were obtained from patients who had been treated with chemotherapy and eleven were from patients who had not received chemotherapy or had chemotherapy for less than 3 weeks and therefore did not have time to respond. Six of 23 tumors from chemotherapy treated patients had DNA amplification of one of the myc family oncogenes, 4 N-myc and 2 L-myc. None of the 11 tumors from untreated small cell lung cancer patients had any myc family DNA amplification. Nine of the patients had both tumors and tumor cell lines studied. All nine had the same myc family DNA amplification pattern in their tumor cell line and their tumor. Two had N-myc DNA amplification in both the tumor and tumor cell line from the same patient and the other seven had a diploid copy of the myc family gnes in both their tumor and tumor cell line.

Transfection and Expression of a Normal c-myc gene into Non-expression Classic Small Cell Lung Cancer Cell Line Reproduces some of the "variant" Characteristics

c-myc DNA amplification in tumor cell lines has been associated with shortened survival in treated small cell ling cancer and with the more agressive "variant" phenotype in vitro (3). In order to determine if c-myc was causing the phenotypic differences, we recently transfected the c-myc gene into a classic small cell lung cancer cell line which did not express c-myc mRNA (12). For these studies we used a c-myc gene cloned from normal lymphocytes covalently linked to a neo gene was introduced using electoporation into a cloned classic cell line, NCIH209. The c-myc gene was expressed in varing degrees in different transfectants. The clone expressing themost c-myc mRNA had a more linear pattern in cell culture, formed a large cell/small cell efficiency, and shorter doubling times than the original cloned small cell lung cancer cell line not expressing c-myc. In contrast to the changes in morphology and growth characteristics, the biochemical characteristics did not change. Thus, the phenotype of the c-myc transfected clones changed to some of the characteristics of the variant cell lines.

Restriction Fragment Polymorphism Studies Show consistent Deletion of Loci on the Short Arm of Chromosome 3 in Small Cell Lung Cancer Patients' Tumors Compared to Their Normal Tissues

Twenty eight of the 34 patients have had normal tissue available for analysis. In collaboration with Dr. Sue Naylor, we have prepared DNA from normal and tumor tissue from the same patient and analyzed the tumor directly for loss of heterozygous loci on chromosome 3p in the tumor tissue compared to normal tissue in the same patient. She has evaluated the normal tissue DNA for heterozygous loci on chromosome 3p with the D3S2, D3S3, and D1S1, a random fragment mapping to chromosome 3p and chromosome 1 obtained from Mary Harper. The normal tissue had heterozygous loci within chromosome 3p 14-23 in 25 of the 26 DNAs analyzed. The tumor was reduced to homozygosity in one of these loci in 23 of the 25 patients (88%). Thus, by using this restriction fragment polymorphism analysis, the actual tumors show deletion of at least a portion of the short arm of one chromosome 3 in 22 of the 25 patients. This lends support to the idea of a consistent deletion of the short arm of chromosome 3 in small cell lung cancer.

Therefore, the coordinated study of established cell lines, systematic collection of tumors and normal tissues from a defined cohort of small cell lung cancer patients has allowed us to make a nuber of important observations and be able to uniquely study the relationship between tumor celllines and tumors from the same patient and the potential clinical implication of chromosomal deletion and oncogene amplification.

Proposed Course:

The recently published study of the pattern of myc family oncogene amplification in small cell lung cancer evaluated the small cell lung cancer cell line established from 1977 through 1984. Approximately 30 additional cell lines have been started since that period of time. Nearly all of these cell lines have been established from the prospective study of small cell lung cancer patients presented by Dr. Ihde. We propose to systematically grow these new cell lines. using the biochemical parameters to charaterize them including an L-dopa decarboxylase enzymatic assay, creatine phosphokinase isoenzyme analysis, neuron specific enolase, chromogrannin, calcitonin, and gastrin releasing peptide radioimmunoasaay. We also intend to confirm these biochemical assays using RNase protection assays with CK-BB, chromogrannin, calcitonin, and gastrin releasing peptide probes. Using these biochemical assays and molecular genetic analysis as well as the traditional morphological criteria, we hope to continue to characterized and categorized the cell lines into the classic and variant subclasses. The data will be correlated with the clinical course of the patients from whom the cell lines are established to identify potential clinical associations with survival.

myc Family DNA Amplification Studies

The DNA from themost recent 30 cell lines will also be hybridized to fragments of c-myc, N-myc, and L-myc. A preliminary analysis of approximately 15 of these 30 cell lines reveals only one new myc family DNA amplified cell line compared to 13 of 44 previously studied cell lines. Based on our observation of the appearance of these cell lines and their growth characteristics, we postulate the decline in the number of variant cell lines and the decline in the number of myc family DNA amplified lines correspond temporally to the shift in the chmotherapy treatment of our small cell lung cancer patients. The previous CMC-VAP chemotherapy regimen for small cel lung cancer was switched to a VP/Plat regimen in 1983 for extensive stage patients and in 1986 for limited stage patients. Therefore, we propose to systematically analyze the new cell for their myc family DNA amplification pattern and correlated their amplification status with their drug treatment. If this clinical association of myc family DNA amplification with specific drug treatment programs is consistent we can examine this phenomena in an in vitro model. This would involve exposing small cell lung cancer cell lines which are not amplified but express one of the myc family genes to pulses of CMC-VAP chemotherapy drugs in vitro to see if exposure to these drugs could cause DNA amplification. The same cells would be simultaneously exposed to VP-16/Cisplatin to see if these drugs do not induce DNA amplification.

myc Family Expression Studies

The studies of myc family genes in small cell lung cancer patients has only present in a minority of cell lines studies (13/44). We propose analyzing the expression of these genes in small cell lung cancer cell lines and identifying the clinical situations of the patients from whom the cell lines were established. In order to avoid the expense of preparing poly A mRNA on over 30 cell lines from the prospective study, the sensitive technique of RNase protection assays allows us to examine expression of the myc family of oncognes with relatively few calls. We have currently subcloned 150-400 base pair fragments of L-myc, c-myc, and N-myc into the pGEM system and are in the process of preparing RNA for analysis. After the myc family gene expression of each cell line has been studied, the clinical course of the patients from whom the cell lines were established will be reviewed. Adequate numbers of patients from the prospective trials are available, so if specific gene expression is associated with an altered clinical course, survival differences of a large magnitude could be identifiable.

Collecting adequate tumor tissue for drug testing and molecular genetic analysis at diagnosis continues to be difficult. The clinical protocols appropriately make drug testing the first priority of the tumor tissue available. Therefore, little tissue from diagnostic specimens is available for molecular genetic analysis and the abundant tissue available at autopsy continues to be an important source for biologic material. The high molecular weight DNA prepared from autopsy tissue is nearly always adequate for molecular genetic analysis, consequently, we plan to continue to collect tumor specimens and normal

tissue from patients at autopsy. This allows us to study DNA amplification patterns directly in tumor specimens and DNA deletions in tumor compared to normal tissue. This also provides the opportunity for the study of gene expression directly in small cell lung cancer tumors. The adaptation of S1 nuclease and RNase protection assays has allowed us to study mRNA expression of gene products present in relatively small amounts such as gastrin releasing peptide in the tumors obtained. This will allow direct study of mRNA expression of the neuroendocrine markers and myc family members to define relative levels of expression and the clinical situations in which it occurs. Additionally, we have initiated a pathologic reviews of all the autopsy specimens to identify the histologic preservation of the tumor specimen and search for potential histologic associations with gene amplification and expression.

Chromosome 3p Deletions in Small Cell Lung Cancer

Some controversy has existed about the consistency of the 3p deletion observed in cytogenetic studies of small cell lung cancer. The recent identification of three probes that identify restriction fragment polymorphisms in the deleted area of chromosome 3p14-23 had facilitated additional investigation. We have prepared tumor and normal tissue DNA from 28 different small cell lung cancer patients and 11 tumor cell lines established from these same 26 patients at some time during their clinical course. Dr. Sue Naylor is currently examining the normal tissue for restriction fragment polymorphisms with the probes D3S3, D3S2, and Dls1 which map to chromosome 3p14-23. Dr. Naylor is then examining the cell lines and tumor tissue from the same patient to look for loss of one of the heterozygous loci identified in the normal tissue. Dr. Peng is simultaneously karyotyping the 11 tumor cell lines established from the 26 patients to identify abnormalties of chromosome 3p. This data will help clarify whether the same cytogenetic changes are taking place in the patients' actual tumors and cell lines from the same patient. The unique resource of the biologic materials available to us allows verification of these 3p finding in small cell lung cancer patients.

In order to expand our source of easily available normal tissue from small cell lung cancer patients who have cell lines established, we have initiated collecting lymphocytes from all small cell lung cancer patients starting therapy in collaboration with Dr. Zbar of the Immunobiology Branch of the FCRC. Dr. Zbar transforms these cells with Epstein Barr Virus to immortalize the B lymphocytes. This provides a continually available source for normal DNA and cells from untreated patients. These continous lines not only provide a resource for studying of chromosomal deletions, bu are also a valuable resource for studying drug sensitivity. We now have 9 small cell lung cancer cell lines and lymphocyte lines from the same patients allowing us to compare the drug sensitivity pattern of the tumor cells with that of the normal cells from the same patient. We plan to continue to start EBV lines from normal lymphocytes and to store lymphocytes for future use. In addition, these lymphocytes provide a resource for comparing the fragile sites at 3p in small cell lung cancer patients normal lymphocytes to other lung cancer patients and normal individuals.

Pending the results of these studies and the results of other studies of non-small cell lung cancer cytogentics, these techniques can be adapted to collecting material from non-small cell lung cancer patients and initiating analogous studies. In addition, the evaluation of multiple patients with variable breakpoints along chromosome 3 increases the possibility of finding a rearranged band with one of the probes hybidizing to chromosome 3p and thus being able to help localized a potential breakpoint.

Therefore, we believe systematically collecting the unique biologic resources of the NCI-Navy Medical Oncology Branch and studying their properties with a variety of techniques in a structured manner has provided important biologic insights into this disease. As more sophisticated techniques become available this resource will continue to be important. In addition, the availability of clinical data on all patients from whom the cell lines and tumors were obtained and the availability of normal tissue provides unique additional important insights. The devastating effect of lung cancer makes the adaptation of molecular techniques for studies and potential therapeutic adaptation of utmost importance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06595-01 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Clinically Relevant Immunohistochemical Markers in Lung Cancer

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

PI:	Ilona Linnoila, M.D.	Senior Investigator	NCI-NMOB
Others:	James Mulshine, M.D.	Senior Investigator	NCI-NMOB
	Adi Gazdar, M.D.	Senior Investigator	NCI-NMOB

COOPERATING UNITS *(if any)*

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LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Human Tumor Biology Section

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our goal is to define immunohistochemical markers that will best type lung cancer for diagnosis, prognosis, and selection of therapy. Small cell lung cancer (SCLC), characterized by neuroendocrine (NE) features, is responsive to chemo- and radiotherapy. Some non-SCLC also exress NE features. The hypothesis is that these tumors might be more responsive to cytotoxic treatment than other non-SCLC.

A) Characterization of markers - In a retrospective study a comprehensive group of 113 lung cancers were tested for the immunohistochemical expression of 17 antigens using a sensitive avidin-biotin-peroxidase technique. Logistic regression analysis was used to separate tumors into the proper categories (SCLC and carcinoid tumors versus NSCLC) based on the immunohistochemical markers. As a result 95% of the tumors were correctly predicted using the cell counts and staining intensities of only six markers. The results suggested that 1) individual marker counts are not useful in tumor classification, 2) "specific" NE markers such as serotonin and neuropeptides bombesin, calcitonin, ACTH, vasopressin, neurotensin are not useful, 3) the best NE markers are a panel of "general" NE markers (Chromogranin A, Leu 7, NSE) which are present in NE cells throughtout the body.

B) Clinicopathologic correlation - This panel of "general" NE markers were applies to the non-SCLC cases on protocol 83-15 in our branch. There was a concordant expression of immunohistochemical NE markers with other biochemical tests used to characterized NE differentiation such as L-dopa decarboxylase activity. Although the numbers were small, the response rate to chemotherapy was 60% (3/5) in the patients whose tumors were positive for NE markers versus 20% (4/21) in those with negative NE markers.

The significance of the project lies in the possible identification of prognostically important clinical subsets of lung cancer. Immunohistochemistry provides a highly effective and specific technique to achieve this goal.

PROJECT DESCRIPTION

Clinically Relevant Immunohistochemical Markers in Lung Cancer

Professional Staff:

PI:	Ilona Linnoila, M.D.	Senior Investigator	NCI-NMOB
Others:	James Mulshine, M.D.	Senior Investigator	NCI-NMOB
	Adi Gazdar, M.D.	Senior Investigator	NCI-NMOB

Objectives

There are four major histological types of lung cancer, namely small cell lung cancer (SCLC) (25%), adenocarcinoma (25%), squamous cell carcinoma (30%) and large cell carcinoma (15%). For a number of biological and clinical reasons, these lung carcinomas may be divided into SCLC and non-SCLC (NSCLC) tumors. SCLC and the rare bronchial carcinoid express many neuroendocrine (NE) features including dense core granules by electron microscopy, high levels of the key amine producing enzyme L-dopa decarboxylase, and the glycolytic isoezyme neuron-specific enolase (NSE) and hormone or neuropeptide production.

SCLC unlike NSCLC is extrermely sensitive for chemotherapy and radiation, and there are scattered reports on favorable responses to chemotherapy by "atypical endocrine" tumors of the lung. This knowledge together with the recently established NE markers has prompted us to explore if 1) the expression of neuroendocrine markers in NSCLC is associated with favorable response to chemo- or radiotherapy, and 2) if the degree of expression of neuroendocrine markers in SCLC correlates with clinical outcome. Immunhistochemial technique provides a readily applicable tool for this.

Methods Employed

1) Tumors: A comprehensive group of 113 primary lung cancers was chosen from the archives of the departments of pathology at the Bethesda Naval Hospital and National Cancer Institute. In addition, tumor material was obtained also from the patients on NCI protocol 83-15. Serial sections from routinely processed paraffin blocks were used.

2) Antibodies: The application of immunologic techniques that use hormone markers has been hampered by the fact that tumors with similar histologic and cytologic featuresmay produce a variety of immunoreative substaces, and some tumors may sythesize more than one hormone. Recently, a mouse monclonal antibody LK2H10 produced against human pheochromocytoma has been shown to be directed against chromogranin (ChrA) a constituent of secretory granules in most peptide producing endocrine cells. The demonstration of chromogranin in lung tumors serves as a useful marker for a broad spectrum of lung tumors with NE features

including SCLC and the rare bronchial carcinoid. Other general immunohistochemical markers for NE differentiation include monoclonal antibody to Leu-7 (HNK-1). Leu-7 was originally identified in subpopulation of lymphocytes called natural killer cells and later noted to be present also in nerves and wide variety of endocrine cells. Antibodies to NSE also react with nerves and cells of the diffuse NE system and its tumors. The advantage of applying such general NE markers in that they provide a more uniform recognigtion f muliple NE tumors that may in turn synthesize a variety of specific products such as different hormones.

3) Immunohistochemical staining: Staining was performed using the avidin-biotin perotidase technique. Appropriate positive and negative controls were included in each assay. Results of the immunostaining were reviewed scoring both for the intensity of the staining and number of positive cells.

Major Findings

1) Characterization of Markers: We were able to demonstrate that the majority of cells in most SCLC and all carcinoid tumors were positive for the general NE markers and many hormones. Logistic regression analysis was used to separate tumors into the proper categories on the basis of markers and 95% the tumors were correctly classified applying model created from staining indeces of general NE markers (ChrA, Leu 7, NSE). Evaluation of the expression of multiple markers revealed that 7/77 NSCLC had a staining pattern indistiguishable from SCLC.

We have concluded that 1) Application of the general NE markers produces acceptable classification of lung tumors; 2) Most but not all SCLC and carcinoids express multiple NE markers in a high precentage of tumor cells; 3) Occasional NSCLC show staining patterns indistinguishable from SCLC; 4) Many NSCLC contain a small subpopulation of cells expressing NE markers.

2) Clinicopathologic Correlation: The panel of "general" NE markers (ChrA, Leu7, NSE) was applied to the non-SCLC cases on protocol 83-15 ("Treatment of Non-Small Cell lung Cancer Utilizing In Vitro Drug Sensitivity"). Based on a detailed histopathological evaluation of tumor specimens of the patients already entered in the protocol it appears than in over 80% of the cases such an immunohistochemical analysis on untreated patient specimens can be performed. Currently we have stained 87 of the 106 cases entered. The results of 80 cases are summarized in the following table:

GENERAL NE MARKERS IN NSCLC BY HISTOLOGICAL TYPE
(80 CASES ON PROTOCOL 83-15)

(% positive)	Chr A	Leu 7	NSE
Adenocarcinoma	2/45 (4)	10/45 (22)	22/45 (49)
Large cell	6/19 (32)	3/19 (16)	9/19 (47)
Epidermoid	0/11 (0)	2/11 (18)	4/11 (36)
Other	0/2 (0)	0/2 (0)	1/2 (50)
TOTAL NSCLC	8/77 (10)	15/77 (19)	35/77 (45)
Carcinoid	3/3 (100)	3/3 (100)	3/3 (100)

The analysis of the response rate to chemotherapy in non-SCLC patients on protocol 83-15 in correlation with the results of immunohistochemistry revealed a rate 60% (3/5) in the patients whose tumors were positive for NE markers versus 20% (4/21) in those with negative NE markers. There was also a strong correlation of the expression of immunohistochemical NE markers with other biochemical for NE differentiation such as L-dopa decarboxylase levels in tumors.

Significance to Biomedical Research and the Program of the Institute

The significance of the project lies in the possible identification of prognostically important clinical subsets of lung cancer. There are at least 150,000 new case of lung cancer (75% of which are non-SCLC) discovered annually. Our preliminary results support our hypothesis that non-SCLC which express NE features might be more responsive to cytotoxic treatment than other non-SCLC. Immunohistochemistry provides a highly effective and specific manner to screen for these tumors.

Proposed Course

1) The expression of markers will be correlated to the clinical data including performance status, best response, and survival. At the end of the protocol 83-15 we should have accumulated results on 120 patients, if 150 patients are accrued as planned. This will provide a basic correlation.

2) Based on our initial observations we expect that 10-20% of non-small cell lung cancers express neuroendocrine markers. In order to extend the analysis and reach meaningful clinical correlations we have initiated a collaboration with the ECOG (Eastern Cooperative Oncology Group) and LCSG (Lung Cancer Study Group). ECOG and LCSG have full clinical response and survival information on nearly 2.800 treated patients. A large number of these have pretretment tumor samples available for analysis. We plan to study the expression of general NE markers chromogranin, Leu 7 and NSE and relate this to tumor type, response to therapy, and survival.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06596-01 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

In Vitro Drug Testing for Limited SCLC and Phase I Drug Development

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

PI:	Bruce E. Johnson, M.D.	Investigator	NCI-NMOB
Others:	Daniel C. Ihde, M.D.	Investigator	NCI-NMOB
	Adi F. Gazdar, M.D.	Investigator	NCI-NMOB
	John D. Minna, M.D.	Chief	NCI-NMOB

COOPERATING UNITS *(if any)*

Eli Glatstein, M.D., Jane Grayson, M.D., and Karen Strauss, M.D., Radiation Oncology Branch. John Strong, Ph.D., Robert Parker, Ph.D., Biological Chemistry Branch and HooGeung Chun, M.D., Investigational Drug Branch

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Human Tumor Cell Biology and Molecular Genetics and Immunology Sections

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	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.)*

A. A protocol combining twice a day radiotherapy and VP-16/Plat for limited stage lung cancer has been implimented. This protocol also uses in vitro drug testing of the patients own tumor to select chemotherapy agents after week 12. Seven patients have been entered on study and all patients (4/4) who have completed therapy have achieved a complete response and these 4 all continue to be cancer free at 11, 11, 10, and 6 months from starting therapy. No patients have been hospitalized for neutropenic fever or pulmonary toxicity.

B. A phase I trial using dihydrolenperone, an agent identified as being active against human lung cancer by the human tumor colony forming assay (HTCFA) has been initiated. Fifteen patients have been studied at 5 dosage levels The principal side effects have been somnolence and hypotension in all patients. Only one patient has had to stop therapy because of sommolence and none because of hypotension. Preliminary pharmacokinetic determinations show peak absorption at 3 hour and that level vary less than 50% over the 12 hours dosing interval.

From this studies we conclude that the HTCFA has identified a compound with novel side effects and the maximum tolerated dose has not been reached.

PROJECT DESCRIPTION

IN VITRO DRUG TESTING TO SELECT CHEMOTHERAPY REGIMENS TO PROLONG DISEASE FREES Remission and Survival in Limited Stage Small Cell Lung Cancer Patients Treated with Combined Modality Therapy and Identify New Drugs for Treatment of Lung Cancer

Professional Staff:

PI:	Bruce E. Johson, M.D.	Senior Investigator	NCI-NMOB
Others:	Daniel C. Ihde, M.D.	Senior Investigator	NCI-NMOB
	Adi F. Gazdar, M.D.	Senior Investigator	NCI-NMOB
	John D. Minna, M.D.	Chief	NCI-NMOB

Objectives:

1. Determine the frequency with which adequate tumor tissue can safely be obtained and drug sensitivity data determined.
2. Determine the response rate, tocicity, and survival of limited stage small cell lung cancer patients treated with VP/PLAT, simultaneous twice a day chest radiotherapy, and chemotherapy based on intro drug testing or a standard regimen (VAC).
3. Determine changes in drug sensitivity when tumor is obtained pre and post-treatment from the same patient.
4. Determine the side effects and maximum tolerated dose of dihydrolenperone.
5. Determine the pharmacokinetics of orally administered dihydrolenperone.
6. Determine the activity of dihydrolenperone within the confines of a Phase I trial.
7. Determine the correlation between in vitro determined activity of dihydrolenperone and the patient response.

Methods:

A. Study Design

1. Small cell lung cancer patients undergo staging.
2. Limited stage patients undergo sugical biopsy of tumor tissue.
3. Induction with 12 weeks of VP-16/PLAT with concomitant 150 RAD twice a day radiotherapy to 4500 RAD over 19 days.
4. Patients with in vitro drug sensitivity data receive an additional 12 weeks of the in vitro best regimen, patients with no in vitro data receive 12 weeks of a standard vincristine, doxorubicin, and cyclophosphamide regimen.
5. Patients are followed for survival and toxicity.
6. Small cell lung cancer patients failing conventional combination chemotherapy and non-small cell lung cancer patients for whom no curative therapies are available are identified for Phase I drug trial.
7. Patients are treated orally twice a day for 28 days with dihydrolenperone and observed for toxicity.

8. Patients with tumor tissue available have in vitro testing with DHLP performed.

Major Findings:

The limited stage study was approved to enter patients in July of 1986. Six patients have been entered to date and all have completed their course of radiotherapy and three weeks of VP/PLAT. Of these six, one patient has had tumor tissue reach the lab. Two patients who were in tolerable medical condition refused to have a thoracic procedure to obtain tumor tissue. Three patients have been entered on study have not had surgical procedures performed, two because of coronary artery disease leading to coronary artery bypass grafting or myocardial infraction. The third was a seventy year old man who did not have surgery performed because he would have required a lobectomy to obtain tissue.

All three patients who have completed 24 weeks of their therapy have had a complete response, one at 12 weeks and 2 at 14 weeks. The other three have not completed their restaging at 12 weeks. One of the six patients has developed pulmonary toxicity requiring steroid treatment but has not needed hospitalization. Two of the six patients have develped esopageal toxicity requiring hospitalization and intravenous fluid support. No patients have been hospitalized for neutropenic fever. All patients are alive and currently free of disease or still receiving their initial therapy.

The phase I and pharmacokinetic study dihydrolenperone was approved to enter patients in January of 1986. Fourteen lung cancer patients have been entered to date and have completed 14 courses of dihydrolenperone with 2 patients currently being treated. The initial dose was 10mg/m^2 orally twice a day for 28 days. The dosage has been escalated to 20mg/m^2, 30mg/m^2, and currently is 40mg/m^2. The prominent side effects have been hypotension and somnolence in all patients. The hypotension observed in the first two patients treated with 20mg/m^2 was 70/50 when the patients were standing. This prompted us to alter the loading schedule with dihydrolenperone so the dosage was increased 10mg/m^2 twice daily until the target dosage is reached. The hypotension with this schedule has been more tolerable. Somnolence has been noticeable in all patients with more pronounced somnolence in patients taking narcotics. One patient who was taking methadone for pain caused by tumor involving the liver discontinued taking dihydrolenperone because of somnolence causing him to sleep more than half the day. No other patient has stopped taking the drug because of somnolence. One other additional patient developed rather severe depression while taking the drug with a flat affect and inability to initiate activities. This developed over the Christmas and New Years holidays. He finished the course of therapy and the depression continued after he stopped the drug. He is currently on Elavil therapy.

Two additional patients did not complete their prescribed courses of dihydrolenperone. One patient developed nausea and vomiting and his drug was stopped. He died within 3 weeks and his autopsy showed tumor constricting his small intestine leading to an intestinal obstruction. The other patient died suddenly two days after his hospital discharge after starting dihydrolenperone. Autopsy

showed a massive pulmonary embolus as a cause of death. There has been no observable hematologic, pulmonary, renal, or hepatic toxicity at the dosage levels studied.

The pharmacokinetic studies by John Strong of the Biologic Chemistry Branch using HPLC have demonstrated that peak absorption approximately 3 hours after the oral dosage. In addition, the twice a day schedule gives drug levels fluctuating less than 50% during the day. The determination of a half life awaits additional studies at higher dosage levels where the drug can be measured more easily.

The in vitro studies are currently being initiated by Dr. Gazdar to determine the IC 50 of a variety of small cell lung cancer and non- small cell lung cancer cell lines to attempt to identify a drug level which identifies sensitive and resistant levels in vitro.

Ten different patients have completed at least one course of therapy and are available for analysis of response to therapy. One small cell lung cancer patient was treated at 30mg/m^2 and had a minor response with shrinkage of a lymph node. Three non-small cell lung patients previously treated with combination chemotherapy progressed while on therapy and six patients had stable disease.

Proposed Course:

1. Determine the frequency with which adequate tumor tissue can safely be obtained and drug sensitivity data determined.
2. Determine the response rate, toxicity, and survival of limited stage small cell lung cancer patients treated with VP/PLAT, simultaneous twice a day chest radiotherpay, and chemotherapy based on intro drug testing or a standard regimen (VAC).
3. Determine changes in drug sensitivity when tumor is obtained pre and post-treatment from the same patient.
4. Determine the side effects and maximum tolerated dose of dihydrolenperone.
5. Determine the pharmacokinetics of orally administered dihydrolenperone.
6. Determine the activity of dihydrolenperone within the confines of a Phase I trial.
7. Deterime the correlation between in vitro determined activity of dihydrolenperone and the patient response.

Explantion of proposed course:

When we originally designed the limited stage study, our aim was to be able to obtain tumor tissue on two thirds of the patients prior to the initiation of treatment. Unfortunately only one out of our first six initial patients has had an operation to obtain tissue. We believe from previous experience that having only 3 of the first 6 limited stage patients be medically capable of tolerating surgical biopsy of tumor is rather unusual. Two of the first three limited stage patients did not consent to have a surgical resection. We believe that the proportion of patients accepting this biopsy will increase as we gain additional

experience in approaching the patients, the coordination between the surgical team and ward staff improves, and additional information on the safety of the procedure is accumulated.

We also are interested in obtaining drug sensitivity, cell biology, and molecular genetic data on tumor tissue and cell lines obtained from limited stage small cell lung cancer patients. We currently do not have any tumor cell lines established from intrathoracic tumor of limited stage patients prior to the initiation of chemotherapy. We would like to examine these cell lines for their drug sensitivity patterns to see if these tumor cells are more sensitive to chemotherapeutic agents than tumor cell obtained from extensive stage patients similar to the pattern observed clinically (1). The possibility exists that it will be difficult to grow enough cells to do drug sensitivity testing by week 12. Previous studies have shown that extensive stage patients who have accessible tumor tissue obtained, brought to the laboratory, and have a cell line established, live significantly shorter than other extensive stage small cell lung cancer patients. Nearly, all our previous experience in growing small cell lung cancer cells has been from these extensive stage patients. It is possible that when we obtain tumor tissue from these patients with a more favorable prognosis it will be more difficult to obtain sufficient tumor cells to do drug sensitivity testing by week 12. If this proves to be the case, we will focus on getting larger surgical biopsies at presentation.

The complete response rate to combined modality therapy in limited stage small cell lung cancer at most institutions is 50-90%. We don't expect to be able to improve on this rate in the current trial. Our major goal is to maintain the therapeutic advantages of combined modality therapy (chemotherapy plus radiotherapy) and decrease the toxicity. The most serious toxicity encountered in our previous limited stage study was fatal pulmonary toxicity in 5 of 47 patients treated with combined chest radiotherapy and chemotherapy. The current plan is to use the twice a day chest radiotherapy to reduce the pulmonary toxicity. One patient of the first six treated developed pulmonary toxicity requiring steroid treatment.

In addition, we believe limited stage small cell lung cancer is an even more important tumor system to test the in vitro drug testing capabilities versus a standard empiric regimen (VAC) than extensive stage small cell lung cancer. It obviously is not important to identify agents that the tumors are resistant to. The clinical utility is to identify agents the tumor is sensitive to. By testing the in vitro drug sensitivity assay in more responsive limited stage small cell lung cancer, we believe we will be better able to define the clinical usefulness of in vitro assays in this more chemosensitive system.

We continue to study dihydrolenperone in lung cancer patients. The maximum tolerated dose has not yet been reached after four dosage levels. The principal side effects of hypotension and somnolence have not dramatically increased in the last two dosage levels. There have been no major unanticipated side effects with the drug to date. We will continue to accrue patients until we reach dose limiting toxicity.

Dr. Strong has been having some difficulty determining drug levels using an HPLC assays. We currently plan to continue to perfect the assay and do

drug levels at these higher dosages levels. We also intent to measure urinary excretion of the drug from the patients treated at these higher dosage levels.

There has been no clear evidence of antitumor activity in the trial so far. We await further accumulation of data at the higher dosage levels. In addition, we intend totest a wide variety of small cell lung cancer and non-small cell lung cancer cell lines to identify the IC 50 concentration of dihydrolenperone. This will be followed by testing tumor cell lines and tumors of patients who have been treated with dihydrolenperone to compare the in vitro activity to the clinical outcome of the patients from whom the tumors were obtained or the tumor cell line established.

In summary, the current plan is to obtain tumor tissue on as many limited small cell lung cancer patients as possible, treat them with combined modality therapy using twice a day radiation to decrease the pulmonary toxicity, use chemotherapy identified by in vitro testing and follow the paitents for response duration and survival. In addition, we plan to continue treateing as many lung cancer patients as possible with DHLP, the drug identified by an in vitro screen. The relative sensitivities of various tumors and tumor cell lines needs to be identified and then the tumors and tumor cell lines obtained from the patients who received DHLP need to be tested and correlated with disease response. The general mechanism of investigation into new compounds identified by in vitro screening against specific tumors is potentially adaptable to any new compound currently being brought into Phase I trials. We believe our resources here are well utilized in testing of new compounds identified by in vitro screening because the methods for validating the assay in tumors obtained from patients actually treated with the compound are available.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-CM-06597-01 NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Supportive Care Project

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

PI:	James L. Mulshine, MD	Senior Investigator	NCI-NMOB
Others:	Ilona Linnoila, MD	Pathologist	NCI-NMOB
	James Tsai, MD	Research Associate	NCI-NMOB
	Herbert Oie, PhD	Research Biologist	NCI-NMOB
	Edward Russell,	Research Biologist	NCI-NMOB
	Mae Jean Englee	Biology Lab Technician	NCI-NMOB
	Sandra Jensen	Biology Lab Technician	NCI-NMOB

COOPERATING UNITS (if any) Anatomic Pathology, Naval Hospital, Bethesda (J. Cottilingham); Pulmonary Medicine, Naval Hospital, Bethesda, (T Walsh); Thoracic Surgery, Naval Hospital, Bethesda (E. Woods); Radiation Oncology Branch, Surgery Branch, (J. Grayson); Clinical Oncology Program

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Clinical Investigations Branch (Section Head: Daniel Ihde)

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

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.)

A primary objective of this Branch is to improve the state-of-the-art in the therapy of lung cancer. In the past, this Branch had focused this effort in the study of small cell lung cancer. With the advances both in the therapy of the small cell patients as well as in the study of small cell lung cancer biology, we decided to generalize the Branch effort to include the systematic evaluation of non-small cell lung cancer. The vehicle for this pilot study of the feasibility and value of using in vitro criteria to select therapy for patients with metastatic non-small cell lung cancer.

PROJECT DESCRIPTION

Supportive Care Project

PROFESSIONAL STAFF:

PI: James L.	Mulshine, MD	Senior Investigator	NCI-NMOB
Others:	Ilona Linnoila, MD	Pathologist	NCI-NMOB
	James Tasi, MD	Research Associate	NCI-NMOB
	Herbert Oie, PhD	Research Biologist	NCI-NMOB
	Edward Russell,	Research Biologist	NCI-NMOB
	Mae Jean Englee	Biology Lab Technician	NCI-NMOB
	Sandra Jensen	Biology Lab Technician	NCI-NMOB

OBJECTIVES:

1. To improve therapy of non small cell lung cancer by selecting chemotherapy on the basis of in vitro analyses, both of drug sensitivity and neuroendocrine markers. To use that protocol as a vehicle for the in-depth study of small cell lung cancer biology.

2. Pilot study to evaluate if patients treated on the basis of their tumor cells' in vitro response to a panel of chemotherapeutic agents have more effective tumor cytoreduction than conventionally treated control patients or historic controls.

3. To determine if non-small cell lung cancer patients with tumors expressing neuroendocrine features characteristic if small cell lung cancer experience natural history more typical of small cell lung cancer.

4. To evaluate our ability to prospectively establish clinical specimens as long-term cell lines.

   a. Optimizing our ability to grow specimens, especially in developing serum-free media systems.

   b. Use the computerized clinical and laboratory data bases to correlate the in vitro findings with natural history.

METHODS EMPLOYED:

1. Clinical trial

2. In vitro drug sensitivity analysis

3. Immunohistochemistry

4. Biochemical Marker analysis

5. Cell culture

MAJOR FINDINGS:

Since this study opened in April, 1984, over 100 patients have been accrued to this study. We performed an interim analysis for the first 90 patients on the study.

As a function of protocol design, all patients had tumor tissue come to the laboratory. In several instances, the tumor tissue non-viable due to immersion in formaldehyde, but excellent cooperation between surgeons and pathologists resulted in a better than 95% yield. In order to obtain tissue from as many patients as possible, we frequently obtain tissue from patients undergoing potential curative thoracic resection. We treat only those patients with metastatic disease that is measurable or can be evaluated. Of the 35 patients who already received chemotherapy on this protocol, tumor tissue arrived in the lab was of sufficient size and condition to do at least limited in vitro drug sensitivity analysis in 29%. Some patients have relapsed and died without any chemotherapy (5 patients) and many more are still followed without any evidence of recurrent disease (39 patients). We have established continuous cell lines on 23% of the patients we have evaluated. We project that the frequency of successfully performing in vitro analysis with our current approach may increase to 40% of the total prospective cases. Further refinements of this approach will be necessary to permit this approach to be more generally applicable and we will outline some of the research directions we are pursuing to accomplish this.

These cell lines are a very useful recourse in conducting further experiments to improve the frequency of successful drug sensitivity analysis. First, the initial cell lines derived in the course of non-small cell protocol are being used in validation of another technique of drug sensitivity analysis, the semi-automatic colorimetric assay. This work will be discussed elsewhere in this document, but there are two areas in which the work with this assay impacts on the non-small cell clinical trial. First, this assay requires significantly less operator time to perform, has a more objective mode of analysis, and ultimately may require a smaller number of tumor cells for analysis. Due to the efficacy of this technique, we might also be able to achieve the goal of testing combinations instead of single agents in vitro. For these reasons, we are motivated to substitute this assay for the dye exclusion assay, after we determine the degree of comparability between the two assays. Second, we have used this assay to examine the growth factor requirement of small cell lung cancer to optimize a serum-free media system for those cells. We are now ready to extend this approach to non-small cell tumors as it is apparent from our low rate of successfully generating tumor cell lines that our current media systems are suboptimal. Both of these adjustments, a more efficient in vitro assay and a more effective media system, have the potential of improving the biggest shortcoming of this

approach, that is, increasing the percentage of cases that we can successfully test for drug sensitivity in vitro.

As discussed previously, the number of patients actually receiving the combinations of drugs selected by the assay as being most active (based on single agent activity) is small (8 patients). This number will increase since we plan to accrue another 50 patients and as more of the patients, who underwent potentially curative thoracic resection, develop recurrent disease. Nevertheless, the results of the in vitro analysis to date has been informative. In those eight patients, the in vitro analysis suggested their tumors would be minimally responsive. The eight predicted most active combinations resulted in only a half log of cell kill in vitro.

70% of the single agents tested with these 8 tumors were resistant by our arbitrary scale (resulting in less than 50% tumor cell kill). None of these patients had an objective tumor response, but their median survival was five months. The survival rate was a equivalent to the patients who received empiric etoposide/cisplatin. Since we are still dealing with small numbers of patients, we have not evaluated the two groups for the equivalence of prognostic features, so it is too early to conclude anything about the utility of the in vitro drug selection to see if a trend emerges. This study, which is really a pilot effort, will not definitively answer the questions regarding the clinical value of in vitro drug sensitivity analysis, but it will provide a departure point for constructing subsequent clinical trials to further resolve such issues.

One of the most provocative directions explored in this study is the prospective evaluation of the fate of the subset of non-small cell lung cancer without biochemical features of small cell cancer. We have prospectively analyzed cell tissues obtained in this study for the expression of four biochemical features generally felt to be characteristic of small cell. Based on the previous retrospective work in characterizing these biochemical markers, expected this phenomenon would be present in about 15% of clear cut non-small cell lung cancers. Our hypothesis was that the patients with these tumors would respond to their treatments in a fashion similar to small cell lung cancer patients (i.e. a higher response rate). We were able to do at least one biochemical parameter on 71 of 81 adequate tumors (88%). 11% of these specimens had elevated levels of expression of at least one biochemical marker. Seven non-small cell lung cancer parts with neuroendocrine biochemical features were treated with a combination chemotherapy used extensively in the Branch for small cell lung cancer (cytoxan, methotrexate, CCNU, vincristine, adriamycin, procarbazine). The response rate has been 43% for those seven "neuroendocrine" patients versus 11% for the remaining 28 non-small cell lung cancer patients treated to date on this study with corresponding median survival rate of 9 months. versus 6 months. Considerable work has been done with the in vitro characterization of these neuroendocrine non-small cell lung cancer cell lines, especially in regard to their in vitro drug sensitivity. This will be discussed elsewhere in this document.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Lung cancer is the leading cause of cancer mortality in our society. Non-small cell lung cancer which comprises 75% of all lung cancer is universally fatal once it has metastasized. Despite intensive clinical research, no major improvement has occurred in the treatment of disseminated non-small cell lung cancer. To address this the NCI-Navy Medical Oncology Branch has attempted to integrate a systemic effort to study the biology of this cancer in conjugation with an attempt to optimize the best available treatment. This entails testing a patient's tumor tissue in the laboratory for its response to standard chemotherapy agents. Based on the in vitro result, a combination is constructed that represents the most cytotoxic single agents for a particular patient's tumor.

This approach potentially has general merit in attempting to specifically tailor available treatments to the unique biology of a patient's tumor. This approach also insures tumor tissue comes to our laboratory and is potentially available to be established as a continuous cell line. These cell lines are excellent model systems for a variety of laboratory investigations.

PROPOSED COURSE:

Further accrual of patients to the ongoing protocol will continue. More experience with the process of in vitro drug sensitivity testing to select patient therapy is required both to further analysis of its further refine the process as well as allow fuller analysis of its benefit.

Independent validation of the enhanced initial responsiveness to chemotherapy of patients whose tumor empresses neuroendocrine differentiation is required to corroborate the preliminary clinical trial outcome. To accomplish this, collaborations have been developed with two cooperative groups to analyze for the expression of neuroendocrine features from tumor specimens obtained from patients already treated with chemotherapy. The goal would be examine if the correlation over neuroendocrine expression with enhanced responsiveness to chemotherapy. Further associated biological studies will also proceed.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-CM-06598-01-NMOB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Diagnostic and Therapeutic Clinical Trials with Monoclonal Antibodies - Part I

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

PI: James L. Mulshine, MD Senior Investigator NCI-NMOB

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Ilona Linnoila, MD Pathologist NCI-NMOB

Frank Cuttitta, PhD Senior Scientist NCI-NMOB

Daniel C. Ihde, MD Senior Investigator NCI-NMOB

Eric Seifter, MD Investigator NCI-NMOB

Barnett Kramer, MD Senior Investigator NCI-NMOB

Ed Sausville, MD, PhD Investigator NCI-NMOB

COOPERATING UNITS *(if any)*

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LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Clinical Investigation (Section Head: Daniel Ihde)

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	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 efforts of this Branch has been central to the recognition of gastrin releasing peptide as an autocrine growth factor for small cell lung cancer (7). Dr. Cuttitta developed a monoclonal antibody (2A11) to the active portion of that peptide and demonstrated that the immunoglobulin could block the mitogenic effect of GRP in vitro and in vivo. We have recently, in collaboration with Hybritech, Inc. (San Diego, CA), initiated a clinical trial to test whether one can control autocrine mediated malignant proliferation of small cell lung cancer using a monoclonal antibody. Our Branch has a long standing interest in the role of growth factors in cancer, so that information from 2A11 antibody clinical trial could be a foundation from subsequent anti-growth factor trials.

PROJECT DESCRIPTION

Diagnostic and Therapeutic Clinical Trials
With Monoclonal Antibodies
(Part I - Clinical Investigations)

PROFESSIONAL STAFF:

PI: James L.	Mulshine, MD	Senior Investigator	NCI-NMOB
Others:	Adi Gazdar, MD	Senior Investigator	NCI-NMOB
	Ilona Linnoila, MD	Pathologist	NCI-NMOB
	Frank Cuttitta, MD	Senior Scientist	NCI-NMOB
	Daniel C. Ihde, MD	Senior Investigator	NCI-NMOB
	Eric Seifter, MD	Investigator	NCI-NMOB
	Barnett S. Kramer, MD	Senior Investigator	NCI-NMOB
	Ed Sausville, MD, PhD	Investigator	NCI-NMOB
	John Minna, MD	Senior Investigator	NCI-NMOB
	Ingalill Avis, RN	Biologist	NCI-NMOB

OBJECTIVES:

1. To study the pharmakinetics of monoclonal antibody delivery, attempting to maximize delivery of antibody to tumor involved sites.

2. To study methods of radiolabeled monoclonal antibody imaging as a staging tool in evaluating patients with cancer.

3. To determine if monoclonal antibody can be used to block growth factor stimulated tumor proliferation.

4. To study tumor cells to identify other growth factors which are potential targets for immunomolecular regulation.

METHODS EMPLOYED:

1. Radionuclide Imaging

2. Immunohistochemistry

3. Radioimmunoassay

4. Radioautography

MAJOR FINDINGS:

1. This Branch was involved in an early monoclonal antibody therapy

trials in cutaneous T-cell lymphoma to determine if the monoclonal antibody could mediate cytoreduction by enhancing immune clearance of malignant T-cell. This trial failed to demonstrate significant therapeutic benefit, but did provide a vehicle for successful diagnostic imaging studies. The initial diagnostic imaging studies were developed by Paul Bunn, M.D., and continued by Dr. Mulshine. The intravenous delivery of In^{111} labeled T101 has resulted in the highest percent of tumor targeting achieved as of the time of its reporting. This localization efficiency was further therapeutic improved after regional delivery via the lymphatics of subcutaneously delivered In^{111} labeled T101. Further studies included a comparison of quality imaging IN^{131} T101 versus In^{111} T101. In this study, the In^{111} conjugate was clearly superior. This work now proceeds to further analysis of specificity of targeting by using an isotopically matched In^{111}-control antibody in sequential scanning studies with In^{111} T101. Information generated in the course of these studies include enhanced understanding of the pharmacology of antibody targeting, the immunogenicity of administered mouse immunoglobulin, and the efficiency if regional delivery techniques. These studies collectively provide the basis for proceeding with the radiolabeled T101 therapy trial which is discussed separately. Efforts have been productive, both in terms of published manuscripts and in developing useful collaborations with other investigators at the Clinical Center engaged in clinical research with monoclonal antibodies.

2. We have been involved with developing a trial with Coulter Immunology using one of their antibodies called KC-4. KC-4 is a monoclonal antibody generated after immunizing a mouse with fresh human lung cancers. The antibody has limited normal tissue distribution. The antigen is expressed densely on the cell surface of tumor cells and does not modulate. Coulter Immunology has developed a technique for labeling the antibody so that we will have access to an indium ^{111} and yttrium ^{90} conjugates (for imaging and therapy respectively). Class switch variants of KC-4 are available for all immunoglobulin classes. For these reasons, it seemed to be a reasonable candidate for clinical trial. The trial was originally written in 1984, but the trial has not yet been activated. This reflected some development delays on the part of the manufacturer and also the prioritization of Branch resources in order to insure successful initiation of the 2A11 antibody trial.

KC-4 is representative of a broad class of monoclonal antibodies which bind to solid tumor antigen. The availability of sufficient quantities of all isotypes for clinical evaluation with the potential for imaging and ultimately radiolabeled therapy make this project attractive. Our collaborators at the Clinical Center in the Radiation Oncology and Nuclear Medicine Branches are committed to studying the utility of radiolabeled monclonals for therapy and are supportive of using the lung cancer patients as a model for this approach.

3. Small cell lung cancer has been extensively studied both at this Branch and elsewhere as a model of a neuroendocrine tumor. Small cell lung cancer has already been reported to produce over 25 different peptide hormone products. Recently, workers from our lab sequenced the gene for GRP from small cell lung cancer. A family of previously unknown peptides synthesized from open reading frames found on the GRP gene. Hetero anti-sera were developed to the three GRP gene associated peptides (G-Gap peptides). By several assays, immunologic evidence of expression of these three distinct products was documented in both small cell tumors and in fetal tissues. These facts suggest that despite the already known numbers of peptide products of small cell lung cancer, there may be a considerably larger number of small cell tumor products. With the rapid development of many areas of biotechnology, the techniques may now be available to begin systematically evaluating the total peptide synthetic capabilities of small cell.

4. We are interested in elucidating and additional new peptide products of small cell, we propose to focus on those peptide products that possess mitogenic capabilities. To facilitate this, we have invested considerable time in validating a semi-automatic colorimetric assay for evaluation of growth factor effects. The parameters to evaluation for such an application are considerably different than the conditions for the assay as reported by Carmichael and others from our Branch. The advantage of this assay is that it provides the exceedingly efficient assay to monitor for growth stimulatory effects, which will be essential when screening large numbers of purified fractions generated in typical HPLC purification efforts.

5. Using the semi-automatic colorimetric assay, we have already demonstrated the mitogenic effect of insulin-like growth factor-1 (IGF-1) on small cell lung cancer cell lines. We have further demonstrated that this effect can be blocked by a monoclonal antibody specific for the anti-IGF-1 receptor.

   We have done considerable work in exploring the biology of IGF-1 in small cell and it appears to be an attractive candidate to target for a therapy approach similar to the anti-GRP monoclonal antibody trial. In thinking about GRP and a candidate for immunotherapy, the limited role this molecule plays in normal adult physiology potentially permits one to completely block this peptide effect without lethal consequences. The situation with IGF-1 may not be similar as this molecule plays a more obvious role in normal adult physiology. Although that might not prevent us from exploring the same type of anti-autocrine factor strategy we employed in the anti- GRP trial, it did provoke us to consider approaches.

6. Many investigators have suggested that cancer can be thought of as a reexpression of normal embryonic and fetal developmental processes. An extrapolation is that autocrine type stimulation may be an important developmental mechanism. If so, such autocrine proliferation

should be controlled through some signaling mechanism to allow for the uniform development of a fetus. In cancer, autocrine proliferation proceeds unabated either because of the regulatory signal. We tested to see if the stimulation of small cell lung cancer mediated be IGF-1 could be inhibited by glucagon, a normal antagonist of IGF-1 activity. Of interest, at a concentration of 10mg/ml, glucagon inhibits the growth enhancement of exogenous effect of IGF-1 in other cell lines. In addition, we are attempting to define the mechanism mediating the inhibitory effect in the cell lines responsive to glucagon.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

These studies have two goals: First to complete the ongoing trial which represent a first effort to establish the clinical utility of monoclonal antibody based imaging and treatment approaches; Second, we have design ongoing in vitro analysis in conjunction with the clinical trials as well as other laboratory investigations to develop second generation biological trials which lend to more effective therapeutic control of malignant proliferation.

PROPOSED COURSE:

The Phase I component of the anti-GRP trial is ongoing and will be extended to Phase II when appropriate. Clinical trials with the other antibodies will also continue with the goal of moving to radionuclide conjugate therapy using monoclonal antibodies in cutaneous T-cell lymphoma and lung cancer. Further work will continue to develop a feasible approach to block IGF-1 stimulation of lung cancer. Dr. Cuttitta is generating antibodies against synthetic peptides from various portions of prepro IGF-1 and the IGF-1 receptor. Using either an available reagent or Branch derived product, we will do further in vivo work to block IGF-1 stimulation. This work may lead us to a clinical trial in a similar fashion to the anti-GRP monoclonal antibody trial. Ultimately, one may choose to block both GRP and IGF-1 stimulation. Careful analysis of early growth factor therapy trials may provide insight as to how best to proceed.

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4. Mulshine JL, Keenan AM, Carrasquillo JA, et al. Immunolymphoscintigraphy of Pulmonary and Mediastinal Lymph Nodes in Dogs: A new approach to lung cancer imaging. Can Res 1987; 47:3572-3576.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-CM-06599-01 NMOB

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October 1, 1986 to September 30, 1987

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

Supportive Care Project

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

PI:	Barnett S. Kramer, MD	Senior Investigator	NCI-NMOB
Others:	Steven Veach, MD	Capt., MC, USN	NCI-NMOB
	Michael Bolger, MD	Lt. Cdr., MC, USN	NCI-NMOB
	Randy Howard, MD	Cdr., MC, USN	NCI-NMOB

COOPERATING UNITS *(if any)* Division of Infectious Diseases, Naval Hospital, Bethesda (Walter Carney, Kenneth Wagner, Matthew Pollack); Pediatric Oncology Branch, NCI (Philip Pizzo)

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Clinical Investigation (Section Head: Daniel Ihde)

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	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 primary goal of this group is to investigate the prophylaxis and management of infectious complications of cancer patients undergoing chemotherapy. The present study is one of prophylaxis of infection in neutropenic patients with passive immunization. It is a randomized study of intravenous gammaglobulin (IVIG) versus placebo. The kinetics of IVIG is also being studied. The project should soon be completed. The next project is a randomized study of monotherapy (ceftazidime) versus ceftazidime plus vancomycin for the initial empiric therapy of febrile cancer patients with very low white blood counts. This study is due to open shortly

PROJECT DESCRIPTION

Supportive Care Project

PROFESSIONAL STAFF:

PI:	Barnett S. Kramer, MD	Senior Investigator	NCI-NMOB
Others:	Steven Veach, MD	Capt., MC, USN	NCI-NMOB
	Michael Bolger, MD	Lt. Cdr., MC, USN	NCI-NMOB
	Randy Howard, MD	Cdr., MC, USN	NCI-NMOB

OBJECTIVES:

A. IVIG Study

1. To examine in a randomized double blinded study whether passive immunization with intravenous gamma globulin (IVIG) is effective as prophylaxis against infection in neutropenic cancer patients.

2. To determine the pharmacokinetics of IVIG and correlate with efficacy of IVIG.

3. To monitor toxicity of IVIG.

B. Ceftazidime Study

To establish criteria for the addition of vancomycin to initial empiric monotherapy with ceftazidime in the empiric treatment of febrile neutropenic cancer patients.

METHODS EMPLOYED:

Both of the above studies are prospective and randomized. The IVIG study is double-blinded with a placebo arm using albumin.

Assays for blood immunoglobulin levels are being performed by Dr. Mat Pollack in the USUHS Division of Infectious Diseases.

MAJOR FINDINGS:

The IVIG Study is nearing completion. Since it is double-blinded, we do not know the outcome by treatment group. A majority of patients became febrile. At present, the statistician, Dr. Seth Steinberg, is analyzing the data (keeping the results blinded to investigators).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Infection is a major cause of death in cancer patients and is a very common reason for hospitalization. If passive immunization could be used to prevent infection, it would decrease a major cause of morbidity and mortality, and might allow the delivery of higher doses of chemotherapy.

The best therapeutic regimen for febrile neutropenic patients has not yet been established. We are looking for an effective non-toxic regimen.

PROPOSED COURSE:

See major findings

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-CM-07250-01 NMOB

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October 1, 1986 to September 30, 1987

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

New Drug Discovery Project

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

PI: Barnett S. Kramer, MD	Senior Investigator	NCI-NMOB
Others: Adi Gazdar, MD	Senior Investigator	NCI-NMOB
Bruce Johnson, MD	Investigator	NCI-NMOB
Daniel Ihde, MD	Senior Investigator	NCI-NMOB
James Mulshine, MD	Investigator	NCI-NMOB

COOPERATING UNITS *(if any)* Radiation Oncology Branch, DCT (Eli Glatstein), Nuclear Medicine, Clinical Center (J. Carrasquillo); Frederick Cancer Research Program (Joseph Mayo); Southern Research Institute (W.R. Laster); Investigational Drug Branch, CTEP (Michelle Christian, Daniel Hoth)

LAB/BRANCH

NCI-Navy Medical Oncology Branch

SECTION

Clinical Investigation (Section Head: Daniel Ihde)

INSTITUTE AND LOCATION

NCI, DCT, COP, Naval Hospital, Bethesda, MD 20814

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	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 primary goal of this group is to identify new agents of potential clinical use in treating solid tumors. A. major effort over the past year has been in the validation of a new in vitro assay which may be helpful as a preclinical screening model for antitumor agents. The model has been used to predict the clinical activity of 7 chemotherapeutic agents against 11 human colorectal carcinoma cell lines which have been developed in this branch. At present, the predictions made from this in vitro model are being validated in a nude mouse xenograft in vivo model. Since the NCI screening program for new agents is based upon this preclinical in vitro assay, it will be very important to validate the results in an in vivo system.

At present, we are involved in several trials of new experimental therapeutic agents: Dihydrolenperone in lung cancer; a radiolabeled monoclonal antibody (^{90}yttrium-T101) in mycosis fungoides and chronic lymphocytic leukemia. We also plan a trial of 4-ipomeanol in lung cancer and a trial of yttrium-labelled anti-CEA monoclonal antibody in colon cancer.

PROJECT DESCRIPTION

New Drug Discovery Project

PROFESSIONAL STAFF:

PI: Barnett S. Kramer, MD	Senior Investigator	NCI-NMOB
Others: Adi Gazdar, MD	Senior Investigator	NCI-NMOB
Bruce Johnson, MD	Investigator	NCI-NMOB
Daniel Ihde, MD	Senior Investigator	NCI-NMOB
James Mulshine, MD	Investigator	NCI-NMOB

OBJECTIVES:

1. Identification of new compounds for the treatment of solid tumors, especially colorectal carcinoma.

2. Preclinical testing of combinations of drugs to detect synergy.

3. Validation of in vitro chemosensitivity test.

4. Testing new compounds in the clinic for lung and colon cancers.

METHODS EMPLOYED:

1. In vitro chemosensitivity: MTT assay (a tetrazolium-based colorimetric test for cell viability.

2. In vivo validation: test of drugs in nude mouse explants of our panel of human colorectal cancer cell lines.

3. Phase I trials of new drugs in cancer (for example, dihydrolenperone and ipomeanol in lung cancer.

MAJOR FINDINGS:

1. 5-FU was the only one of 7 drugs tested which we predict would be effective in some of our colorectal cell lines (see attached manuscript).

2. Leucovorin enhanced the cytotoxicity of 5-FU and of FUdR in 10 of 11 colorectal cell lines tested.

3. The dihydrolenperone studies proceeding (see Dr. Bruce Johnson's Annual Report).

4. The ipomeanol study has not yet opened, nor has the ^{90}yttrium-T101 study.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

New drug development is a major charge of the National Cancer Institute. The preclinical screening program of the NCI is based upon the MTT assay. Here, validation of the assay is critical. It is important also to pursue innovative therapies, such as treatment with radiolabeled monoclonal antibodies directed against malignant cells (e.g. ^{90}yttrium-T101).

PROPOSED COURSE:

We plan to extend our in vitro screening process by developing an in vitro "therapeutic index". Since the dose-limiting toxicity of most anti-neoplastic agents is myelotoxicity, we plan to compare cytotoxicity of drugs against tumor cell lines to that of normal bone marrow progenitor cells.

PUBLICATIONS:

See attached manuscript

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06830-17 PB

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Infectious Complications of Malignancy: Diagnosis, Management and Prevention

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

PI:	Philip A. Pizzo	Head, Infectious Disease Section; Chief	PB, NCI
Other:	J. Hathorn	Senior Staff Fellow (through April)	PB, NCI
	J. Falloon	Senior Staff Fellow	PB, NCI
	E. Albano	Special Volunteer (Children's Hospital, DC)	PB, NCI
	M. Rubin	Special Volunteer (Cornell University)	PB, NCI
	T. Walsh	IPA (University of Maryland)	PB, NCI

Continued on next page

COOPERATING UNITS *(if any)*

Medicine Branch, Surgery Branch, NCI; Diagnostic Microbiology, CC; Bethesda Naval Hospital, University of Miami, Duke University

LAB/BRANCH

Pediatric Branch

SECTION

Infectious Disease

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	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.)*

Our studies are devoted to developing methods to define cancer patients who are at risk for developing serious infection, to improving the ability to diagnose these infections early, to treat them effectively, and ultimately to prevent them. We are developing new therapeutic approaches based on the availability of new antibiotics, particularly the β-lactams and the quinolones. We have shown that certain β-lactams used as single agents can replace the need for combination antibiotic therapy. Our studies are also defining the appropriate antibiotic therapy for documented infections, particularly the role of oral antibiotic therapy; the necessary duration of empiric therapy for patients with unexplained fevers and the choice of empiric antifungal therapy. In addition, we are studying the best type of indwelling catheter to use in the cancer patient and how to manage their related side effects.

We have developed a unique model for studying the pathophysiology, natural history, treatment and prevention of invasive candidiasis in the neutropenic host. This model permits the testing of new antifungal agents as well as immunoregulatory agents. To prevent infections we are evaluating the role of passive immunization with a pooled immunoglobulin preparation that contains activity against the Enterobacteriaceae as well as the pseudomonads. We are also studying other immunoregulatory agents that may serve as adjuncts to the treatment of infection, including interleukin 1 and 2, GM-CSF, G-CSF and M-CSF.

We have initiated Phase I trials with the anti-retroviral agent, azidothymidine for children with AIDS or ARC.

GPO 914-916

Professional Personnel (Continued):

D. Callender	Medical Staff Fellow	MB, NCI
J. Eddy	Clinical Nurse Specialist	CC
J. Gress	Guest Researcher	PB, NCI
D. Marshall	Clinical Nurse Specialist	PB, NCI

Clinical and Laboratory Studies

1. We completed a prospective randomized trial comparing monotherapy with a third-generation cephalosporin to combination antibiotic therapy β-lactams and an aminoglycoside) for the empiric management of febrile neutropenic patients. This trial demonstrated that monotherapy was exactly comparable to combination therapy for the initial empiric management of patients who become febrile while neutropenic, an observation which suggests that synergistic therapy is not necessary if a single agent provides broad-spectrum activity and high serum bactericidal levels. This offers new approaches to therapy that are both practical and cost effective.

   To test this further, we have initiated another randomized trial, this time comparing monotherapy with a third-generation cephalosporin to the new class of antibiotics, the carbapenems. Over 180 patients have been randomized in this study and to date, the results appear comparable. This study is in progress.

2. Our studies of empiric antibiotic therapy have also placed in perspective the appropriate utilization for the aminoglycosides and vancomycin, pointing out that although helpful, these agents are really only necessary in approximately 15% of cancer patients who become febrile and neutropenic.

3. In a randomized trial, we have demonstrated that an oral imidazole, ketoconazole, is as effective as amphotericin B in preventing the emergence of systemic fungal infections in cancer patients who remain persistently febrile while receiving broad-spectrum antibiotic therapy.

4. In a randomized trial, we have demonstrated that it is appropriate to continue empiric antibiotic therapy for a limited (i.e., 2 week) course for patients who have defervesced following the initiation of antibiotics but who remain persistently febrile.

   In a follow-up study, we are comparing the use of a new class of oral antibiotics, the quinolines, for patients who have defervesced on parenteral therapy, have no defined site of infection, and remain persistently granulocytopenic. This study has considerable importance, since it can serve to re-define the role of inpatient versus outpatient therapy for treating the infectious complications that occur in tandem with cancer therapy. To date, 40 patients have been randomized.

5. We have demonstrated the importance of anaerobes in contributing to perianal cellulitis and of the early initiation of specific anti-anaerobic therapy in diminishing the progression of these infections and limiting the need for surgical intervention.

6. To decrease the frequency of infectious complications associated with indwelling intravenous catheters of the Hickman-Broviac type, we have initiated a randomized trial to compare the Hickman-type catheter to a subcutaneously implanted catheter (Port-A-Cath). Since the subcutaneous catheter requires less manipulation, our hypothesis is that it will have a lower incidence of infection. However, if infected, it is possible that these infections will not be capable of eradication unless the catheter is removed. To date, more than 76 patients have been randomized and this study is still in progress.

7. To reduce the incidence of infection in patients who have protracted neutropenia, we are evaluating, in a double-blind randomized trial, the value of passive immunization with a pooled intravenous immunoglobulin. We are also assessing the utility of this immunoglobulin in attenuating the types of infections which occur. To date, over 79 patients have been randomized and this study is still in progress.

8. We have evaluated the role of various opiates and enkephalins on leukocyte function and, in contrast to what has been reported in the literature, we have not demonstrated any adverse effects on chemotaxis or bactericidal activity.

9. We have developed a unique model of candidiasis that closely mimics the pattern of infection that occurs in patients with prolonged periods of neutropenia. This model utilizes rabbits that are maintained in a neutropenic state for 3-4 weeks. This model permits study of the pathophysiology and natural history of invasive fungal infection in a profoundly neutropenic host. It lends itself to the study of various therapeutic and preventative strategies as well as the role of immunoregulatory agents as adjuvants. This model is particularly important in view of the increasing prominance that fungal infections are playing in compromised hosts and the difficulty that exists in studying them. We have also developed models of fungal myositis, endophthalitis and catheter infections.

10. In our neutropenic rabbit model, we have domonstrated the unique pathological lesion that characterizes the syndrome of hepatic candidiasis. This syndrome has only been appreciated during the past several years and we have reviewed the cases that have presented to the NIH, compared them to the world's literature and compared them to the lesions that occur in the experimental model. This will provide insight into diagnosis and therapy and eventually, prevention.

11. In our neutropenic rabbit model, we have evaluated all the antifungal agents that are currently available, as single agents and in combination. We have shown that the kidney is the most difficult organ from which to eradicate infection. In this model, only the combination of amphotericin

B plus 5-fluorocytosine is effective in eliminating infection. We have also evaluated itraconazole and fluconazole in models of sublethal infection, lethal infection, hepatosplenic candidiasis and preventive therapy.

12. We have evaluated the antifungal role of IL-2 and IL-2 plus LAK cells on Candida in an in vitro system and in our neutropenic rabbit model. We are unable to show that these cells or high doses of IL-2 have fungicidal activity.

    We are evaluating GM-CSF, IL-1, M-CSF as adjuncts to therapy in in vitro and in vivo fungal systems.

13. We have evaluated the effect of amphotericin B on the chemotaxis human natural killer cells, monocytes and polymorphonuclear cells and have shown that while monocytes and PMNs are inhibited, NK cells are not if the preparation is free of deoxycholate.

14. We have initiated two phase I studies of antiretrovial therapy with azidothymidine (AZT) in children with the Acquired Immunodeficiency Syndrome (AIDS). One is an intermittent bolus administration of AZT and this study is being done collaboratively with the University of Miami and Duke University. The second is a continuous infusion of AZT using a portable delivery device. Nine patients have been entered in this latter trial with encouraging results to date, including weight gain, decrease in lymph nodes and hepatosplenomegaly, increased T4 cells and significant improvement in neurological function.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06840-12 PB

PERIOD COVERED

October 1, 1986, to September 30, 1987

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

Treatment of Acute Leukemia

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

PI:	David G. Poplack	Head, Leukemia Biology Section	PB, NCI
Others:			
	F. Balis	Investigator	PB, NCI
	C. Arndt	Investigator	PB, NCI
	R. Heideman	Investigator	PB, NCI

COOPERATING UNITS *(if any)*

Metabolism Branch, NCI (S. Korsmeyer); Clin Pathology, NCI (L. Neckers); Navy, NCI (L. Kirsch); Children's Cancer Study Group (G. Reaman).

LAB/BRANCH

Pediatric Branch

SECTION

Leukemia Biology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20892

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.)*

Clinical research into the biology and treatment of acute leukemia is pursued with particular emphasis on acute lymphoblastic leukemia (ALL) of childhood. Major issues being addressed include: 1) development of therapeutic strategies aimed at improving overall prognosis of children with ALL, 2) investigation into the mechanisms of treatment failure with particular emphasis on evaluation of pharmacologic approaches to leukemic therapy, and 3) characterization of adverse sequelae of antileukemic therapy and design of treatment regimens which avoid them.

The major ALL treatment protocol has successfully demonstrated that high-dose, protracted systemic methotrexate infusions can substitute for cranial radiation as central nervous system (CNS) preventive therapy for the majority of patients with ALL. Moreover, analysis of data derived from this study has identified a patient group at particular risk for CNS relapse. A new, high risk protocol has been devised in an attempt to improve the prognosis for these and other poor risk patients. The results to date indicate that this therapy is highly effective in preventing both systemic and central nervous system relapses while avoiding the use of cranial radiation. In patients in the average risk category, a comparison of two forms of CNS preventive therapy (intrathecal vs High Dose Methotrexate) is under way. A major, multi-institutional pharmacologic monitoring protocol has been instituted in an attempt to study the relationship between the bioavailability of orally administered maintenance chemotherapy and relapse in children with ALL. On the basis of in vitro studies indicating that Interleukin-2 will induce phenotypic and functional maturation in acute lymphoblastic leukemia cells, a Phase I study of this potential antileukemic approach is being instituted. Detailed analysis of the immunologic and molecular phenotype of acute lymphoblastic leukemia has led to the concept of a hierarchy of differentiation for both T cell and pre-B cell ALL.

Objectives

1. To develop effective therapeutic regimens in acute lymphoblastic leukemia which provide maximum tumor cell kill and improve the prognosis of children with acute lymphoblastic leukemia.

2. To study the pharmacologic approaches to leukemia therapy in an attempt to probe the pharmacologic reasons for treatment failure in this disease.

3. To evaluate the short- and long-term effects of antileukemic therapy on growth, development and organ function, with particular reference to the central nervous system.

Methods and Major Findings:

A. Treatment Studies of Acute Lymphoblastic Leukemia

1. NCI 77-02/CCG 191 Treatment Protocol

A randomized protocol investigating the efficacy of high dose intravenous methotrexate infusions as CNS preventive therapy. Patients received either cranial radiation plus intrathecal methotrexate or high dose 24-hour intravenous methotrexate infusions. One-hundred-seventy-seven (177) average and high risk patients were randomized on this study. The overall remission rate is 98%. The continuous complete remission rate is approximately 70% at three years for the entire study group. With a median duration on study of 64 months, there is no significant difference in the CNS relapse rate for either treatment group.

2. NCI 83-P/CCG 134P

The major aim of this pilot protocol is to demonstrate that high risk patients can be effectively treated on a regimen that uses CNS preventive therapy devoid of cranial radiation. To date, 66 patients have been entered on study; all achieved complete remission. With a median duration on study of 21 months, the event free survival (at 12 months) is 83%. The occurrence of isolated CNS relapse in only one patient, to date, suggests that effective CNS preventive therapy can be achieved without the use of cranial radiation in high risk patients.

3. NCI 84-A/CCG 144

This protocol randomizes average risk patients to one of two forms of CNS preventive therapy - either high dose methotrexate infusions or intrathecal methotrexate alone. One hundred fifteen patients have been randomized on study. With a median potential duration on study of 15 months, there is no significant difference in the CNS or bone marrow relapse rate in either treatment arm. The event free survival at 12 months is approximately 90%.

B. Pharmacologic Approaches to Leukemic Therapy: Relationship to Treatment Failure

A detailed study of the bioavailability of the major orally administered antileukemic agents is being undertaken in an attempt to examine the reasons for treatment failure in children with ALL. This study will attempt to correlate the results of prospective periodic pharmacokinetic bioavailability studies of 6-MP and methotrexate with relapse rate and remission duration in a multi-institutional setting. Approximately 70 patients have been entered to date.

C. Molecular Biology of Acute Lymphoblastic Leukemia

Collaborative studies are investigating the status of immunoglobulin gene rearrangement and T-cell receptor gene status in acute leukemic lymphoblasts. Studies to date have enabled us to construct a hierarchy of differentiation for both pre-B cell precursor ALL (by immunoglobulin gene rearrangement) and for T-cell rearrangement (using T-cell receptor gene rearrangement).

D. Interleukin-2 as potential therapy for Acute Lymphoblastic Leukemia

In vitro studies with IL-2 have demonstrated its ablity to induce phenotypic and functional maturation in a subset of acute leukemic lymphoblasts. This observation has led to the development of a Phase I trial in patients with hematologic malignancies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06880-10 PB

PERIOD COVERED

October 1, 1986, to September 30, 1987

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

Clinical Pharmacology

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

PI:	David G. Poplack	Head, Leukemia Biology Section	PB, NCI
Others:			
	F. Balis	Investigator	PB, NCI
	C. Arndt	Investigator	PB, NCI
	R. Heideman	Investigator	PB, NCI
	J. Collins	Senior Investigator	CPB, NCI

COOPERATING UNITS *(if any)*

Dept. of Pediatrics, Children's Hospital of Los Angeles (J. Holcenberg); Pharmacy Department, CC, NIH (P. Narang); Children's Hospital National Medical Center (G. Reaman); Children's Hospital of Philadelphia (R. Packer).

LAB/BRANCH

Pediatric Branch

SECTION

Leukemia Biology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

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

The clinical pharmacology of antineoplastic agents used in the treatment of pediatric malignancies is studied with emphasis on the role of pharmacologic monitoring and on both pre-clinical and clinical pharmacologic studies of Phase I agents. The clinical pharmacology of orally administered antileukemic agents has been evaluated and the limited bioavailability and variable drug levels of 6-MP achieved following oral administration has been documented. Studies are underway to determine the extent to which this phenomenon is the cause of treatment failure. Additional efforts to optimize 6-MP administration have been based on in vitro studies which have demonstrated a need for prolonged exposure to cytocidal concentrations of drug to maximize leukemic cell kill. Clinical Phase II protocols evaluating prolonged intravenous 6-MP infusions for acute leukemia, brain tumors and solid tumors are underway. Preclinical and clinical pharmacokinetic studies of the two new agents, Trimetrexate and Spiromustine have been completed. New Phase I studies of are in progress (including 5-FU leucovorin, 5-Azacytidine High Dose Ara-C and Fludarabine phosphate) as is a Phase II study of the Methotrexate-5 FU combination.

A major effort of this project is to study experimental approaches to the treatment of both meningeal and non-meningeal CNS malignancy. A unique sub-human primate model which allows sterile, repetitive access to cerebrospinal fluid, is utilized to study the CNS pharmacokinetics of various intrathecally and intravenously administered chemotherapeutic agents; to evaluate the neurotoxicities attendant upon various CNS treatments; and to evaluate and screen in a pre-clinical setting newer CNS treatment modalities and drug schedules. Information gained from the studies with this model is then applied to the design of clinical treatment protocols. Clinical studies of intrathecal AZQ and intrathecal 6MP are in progress. Pre-clinical studies evaluating intra-CSF drug administration via indwelling drug delivery devices is under way.

GPO 914-918

Objectives:

1. To perform pre-clinical and clinical pharmacologic studies on new agents with particular emphasis on those being used to treat pediatric malignancies and those with potential activity against CNS malignancies.

2. To explore a subhuman primate model which provides repetitive access to the cerebrospinal fluid and allows detailed study of the pharmacology and neurotoxicity of chemotherapeutic agents used to treat CNS malignancy.

3. To study the CNS pharmacokinetics of currently employed and potentially useful CNS antineoplastic agents.

Methods Employed and Major Findings:

A. Clinical Pharmacology of Antineoplastic Agents

1. Studies with 6-Mercaptopurine

Extensive preclinical and pharmacokinetic studies of 6-mercaptopurine (see Project Numbers Z01-CM-06840-09-PB and Z01-CM-06880-08PB) led to the development of current Phase II Studies evaluating the efficacy of prolonged intravenous 6-MP infusions for 1) acute lymphoblastic leukemia, 2) brain tumors, and 3) pediatric solid tumors. These studies are in progress.

2. Clinical Studies of Trimetrexate

Preclinical studies detailed in the previous report (see Project Number Z01-CM-06880-08-PB) led to the development of a Phase I Study of this compound. Forty patients were entered on study. A maximally tolerated dose of 110/mg/m^2 was identified for the weekly times 3, intravenous schedule.

3. Phase I Study of Spirohydantoin Mustard in Pediatric Malignancies

We have completed a Pediatric Phase I Study of Spirohydantoin mustard (spiromustine). A total of 23 patients have been entered on this weekly times 3, intravenous dose schedule. The maximally tolerated dose of Spiromustine in pediatric patients was 9.5 mg/m^2. Pharmacokinetic and protein binding studies suggest that clinically insignificant antineoplastic levels of spiromustine are found in the CNS at clinically tolerable doses.

4. Phase I Studies of Fludarabine Phosphate, 5 Azacytidine/High Dose Cytosine Arabinoside and 5FU-Leucovorin

These three phase I studies are currently in progress.

5. Phase II Study of Methotrexate/5-FU Combination

A Phase II Study of this combination is in progress.

6. Phase I/II of Intrathecal AZQ

Based on work in our subhuman primate model, a Phase I/II Study of Intrathecal AZQ has been conducted and is still in progress. Results to date have been encouraging. A total of 21 patients have been treated. There have been 9 complete responses and 7 partial responses of the 17 evaluable patients. This agent obviously has demonstrated activity against malignant meningeal disease.

7. Phase I Study of Intrathecal 6-MP

Based on studies performed in our subhuman primate model, we have demonstrated the feasibility of intrathecal administration of 6-mercaptopurine. A Phase I Study has been initiated. Of the 8 patients entered on study to date, 2 have achieved a complete response.

8. New Phase I Studies

New Phase I studies of Interleukin-2, intravenous Thiotepa, intravenous Ara-AC and oral piritrexim are currently being initiated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06890-08 PB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Lymphoma Biology and Epstein Barr Virus

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

PI:	Ian T. Magrath	Senior Investigator	PB, NCI
Others:	Jacqueline Whang-Peng	Senior Investigator	MB, NCI
	Ilan Kirsch	Senior Investigator	NMOB, NCI
	Greg Hollis	Senior Investigator	NMOB, NCI
	Jeffrey Moore	Chemist	IR CHB, NHLBI

Continuated on next page

COOPERATING UNITS *(if any)*

Flow Cytometry Laboratory, George Washington University (O. Alabaster); Wistar Institute (C. Croce); Department of Pathology, New York University (R. Dalla-Favera)

LAB/BRANCH

Pediatric Branch

SECTION

Lymphoma Biology Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.75	3.75	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.)*

Our major goal is to understand the pathogenesis of undifferentiated lymphomas in terms of the environmental factors predisposing to these diseases and also the molecular changes responsible for neoplastic behaviour. We have focused predominantly on a comparison of Equatorial African and North American Burkitt's lymphoma.

We have embarked upon an extensive characterization (immunologic and molecular genetics) of a series of cell lines derived predominantly from patients treated in the PB. We have focused particularly on the breakpoints related to the specific chromosomal translocations associated with these tumors, and the regulation of the c-myc gene, an oncogene adjacent to the breakpoint (or including the breakpoint) on chromosome 8.

A number of systems have been established in which expression of either c-myc or the Ig μ gene is affected. These systems are used to gain information regarding the possibility that the c-myc gene is regulated by sequences present in the Ig locus, and brought into proximity with c-myc by virtue of the translocation. We have recently focused upon a comparison of effects on the translocated and normal c-myc alleles in all cell lines which express both genes. These can be differentiated in some cases because of structural differences.

We are studying the regulation of immunoglobulin gene expression in Burkitt's lymphoma cells, especially in the context of allelic exclusion.

We have established a system to examine c-myc regulation by anti-sense oligomers.

GPO 914-918

Professional Personnel (Continued):

J. Sandlund	Biotechnology Fellow	PB, NCI
J. Kiwanuka	Visiting Fellow	PB, NCI
F. Barriga	Medical Staff Fellow	PB, NCI
B. Shiramizu	Medical Staff Fellow	PB, NCI
M. McManaway	Biotechnology Fellow	PB, NCI
D. Nelson	Senior Investigator	MET, NCI
D. Wagner	Medical Staff Fellow	MET, NCI
M. Cheah	Medical Staff Fellow	LMCB, NCI

Major Findings and Future Plans:

A. Molecular Genetic Differences Between African and American Burkitt's Lymphoma

With members of the department of pathology at NYU, we have identified differences in the molecular changes between African and North American Burkitt's lymphoma. In the former, the breakpoint on chromosome 8 is some distance 5' (upstream) of the c-myc gene, while in North American Burkitt's lymphomas, it is either in the 5' flanking sequences, or within the 5' region of the the gene itself. This also correlates (in cell lines) with the secretion of IgM, and confirms our contention, based on clinical findings, that Burkitt's lymphoma from these two areas differs biologically, probably because the cell of origin differs. This further implies a pathogenetic difference between the two tumors. We have recently extended this work to more than 50 fresh tumors from Africa and the USA.

B. Demonstration of Abnormal Expression of c-myc in Burkitt's Lymphoma Cell Lines

In spite of the well described structural abnormalities of the c-myc gene in Burkitt's lymphoma, no clear cut abnormality of its expression has been documented. It has been postulated that the gene is expressed inappropriately. We have obtained direct evidence that this is the case by inducing differentiation in Burkitt's lymphoma cell lines. In this circumstance we have shown that c-myc expression is down-regulated in non-neoplastic B-cell lines, as is the case in a large number of other systems in which differentiation is induced. However, in Burkitt's lymphoma cells, c-myc expression persists unchanged. This is consistent with the persistent expression of Ig genes in differentiating B cells, and also with the possibility that c-myc expression is regulated by Ig sequences. Another model we have utilized, in which anti-Ig down regulates both c-myc and Ig, also supports this explanation. These studies are being extended by examining expression of the normal and abnormal c-myc alleles in the few cell lines which express both.

C. Influence of Cytotoxic Drugs on c-myc Expression

We have shown that several chemotherapeutic agents will cause lowering of cytoplasmic steady state levels of c-myc. This occurs in spite of continued expression of the Ig μ gene, and beta-2-microglobulin. This appears,

at first sight, to conflict with the notion that c-myc expression is induced by Ig sequences. However, it is likely that the down-regulation of c-myc in this circumstance is achieved by a post-transcriptional mechanism. This indicates, in turn, that chemotherapeutic agents can selectively inhibit gene expression, and provides a new perspective on cancer chemotherapy, as well as providing a convenient tool for the regulation of some genes. Further exploration of this phenomenon is planned, specifically by using the nuclear run-off method of differentiation between transcriptional and post-transcriptional regulation.

D. Multiple Heavy-chain Expression in Burkitt's Lymphoma Cell Lines

We have been exploring the multiple expression of Ig heavy chains in Burkitt's lymphoma cell lines and have found several examples of this. Our current hypothesis is that American Burkitt's lymphoma represents a cell at a point immediately prior to Ig switching, at which time it has been shown, there is expression of several heavy chains simultaneously. We plan to further explore this phenomenon, and relate it to the breakpoint sites. It is probable that the translocation in American tumors utilizes enzymes involved in Ig switching, whereas that in African tumors utilizes enzymes involved in VDJ joining. If this is the case, we would anticipate finding a high frequency of breakpoints in the Ig J region in African Burkitt's lymphomas and switch breakpoints in American tumors. Furthermore, the possibility exists that there could be polyclonality at the Ig L locus in African tumors, but not in Americans (see below). These issues are being intensively investigated.

E. Multiple Light-Chain Expression in Burkitt's Lymphoma Cell Lines

We have studied Ig light chain expression in Burkitt's Lymphoma cell lines at the level of RNA and protein by double fluorescence labelling in a flow cytometer, by ELISA studies utilising μ, λ and κ capture and detection, and by Northern blots. We have studied a number of sub-clones of BL lines and believe that we have good evidence that allelic exclusion is not operative in Burkitt's Lymphoma cell lines. Whether there is true polyclonality at the light chain locus remains to be determined. This raises numerous questions regarding the regulation of Ig gene expression in normal and BL cells, and also has implications for the timing of the translocation in relation to cellular differentiation.

F. Anti-sense Regulation of C-myc Expression

We have established a system for assaying the effect of anti-sense c-myc oligomers on BL cell lines. Preliminary results show inhibition of both c-myc translation and cellular proliferation. The system will be optimalized, but has potential for the exploration of the precise effects of c-myc expression in BL cells, including its possible effect (primary or secondary) on cellular differentiation. This approach may have therapeutic implications, especially if we can develop anti-intron oligomers which are specific for sub-sets of translocated c-myc genes.

G. A Burkitt's Lymphoma Xenographt Model

We are establishing nude mouse models of BL xenografts for the further exploration of the influence of α - μ and possibly anti-sense oligomers on the growth of Burkitt's Lymphoma in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06813-05 PB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Molecular Biology of Pediatric Tumors

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

PI:	Mark A. Israel	Head, Molecular Genetics Section	PB, NCI
Others:	L. Helman	Biotechnology Fellow	PB, NCI
	C. Thiele-Galetto	Senior Staff Fellow	PB, NCI
	P. Cohen	Medical Staff Fellow	PB, NCI
	R. LaRocca	Medical Staff Fellow	MB, NCI
	M. Cooper	Medical Staff Fellow	MB, NCI
	T. Kidowaki	Guest Researcher	

COOPERATING UNITS *(if any)*

University of PA, Philadelphia (B. Emanuel, R. Packard, A. Evans); Fordham University, New York (R. Ross); Johns Hopkins University, Baltimore (M. Levine)

LAB/BRANCH

Pediatric Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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.)*

Using human neuroblastoma as a model system, we are studying the molecular events associated with the development of several tumors. Our current focus is to identify and characterize the molecular mechanisms important for the regulation of tissue-specific differentiation and the arrest of growth which invariably accompanies it. In this regard, ongoing work is testing the hypothesis that amongst histologically-indistinguishable solid tumors there are different genetic entities which correspond to cells at different stages in the differentiation of the tissue in which solid tumors arise. Such a schema has been hypothesized for both lymphoid and hematopoietic malignancies in which specific markers of differentiation have been extensively characterized.

During the past year we have sought profiles of gene expression which distinguish amongst histopathologically identical tumors, and we are characterizing the relationship of these different tumor types to different stages in the differentiation of the peripheral nervous system. Experiments to determine whether genetically similar tumors have a homogeneous and predictable biologic behavior are also being pursued.

HS 6040 (Rev. 1/84)

GPO 914-916

Accomplishments and Results:

1. Characterization of alterations in gene regulation which might be central to the development of solid tumors.

Our studies examining the tumor-specific regulation of genes has focused on two themes: 1) altered regulation of genes likely to be important for the development of malignancy and 2) identification of patterns of expressed genes which characterize specific tumors.

We have developed batteries of cloned DNA molecules which identify mRNA species apparently expressed during different stages of neural crest development. We have used such cloned cDNAs to develop molecular fingerprints which we believe will provide a new and uniquely rational basis on which to classify tumors. To date, our work has focused on the characterization of tumors whose tissues of origin is thought to be well known allowing us to generate a series of fingerprints which define both the tissue of origin of a tumor and genetically distinct subgroups amongst histologically indistinguishable tumors. In these experiments we have been able to determine that among solid tumors such as, neuroblastoma, glioma, and colon cancer, there may be multiple pathologic entities which correspond to clonal expansions of cells at different stages of tissue differentiation. In the future, it should be possible to use such reagents to characterize tumors whose tissue of origin or cell of origin within a given tissue is unknown. Also useful in this regard have been proto-oncogenes whose expression has been shown to be related to such biologically important features of tumors as proliferation, tissue lineage and differentiation.

2. Identification and characterization of genes important for growth and tumor cell differentiation.

To identify and characterize genes important for the development of malignancy, we have devised a strategy to examine the possibility that tumors arise from cells which are growing inappropriately because their ability to mature and begin senescence has been compromised. In evaluating panels of genes which characterized either mature chromaffin tissue or malignant neuroblastoma, we determined that the expression of these genes could be modulated by a variety of agents with therapeutic potential. We are currently studying the regulation of these genes in order to determine whether regulatory mechanisms important for tissue-specific differentiation might be pathologically disturbed in tumors of embryonal neuroendocrine tissues, halting the progression of such embryonic tissue to form mature chromaffin tissue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06815-05 PB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Investigation and Treatment of Patients with Non-Hodgkin's Lymphoma

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

PI:	Ian T. Magrath	Senior Investigator	PB, NCI
Other:	Philip A. Pizzo	Chief	PB, NCI
	David G. Poplack	Head, Leukemia Biology Section	PB, NCI
	Mark A. Israel	Head, Molecular Genetics Section	PB, NCI

Continued on next page

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pediatric Branch

SECTION

Lymphoma Biology Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Patients have now been admitted to the primary protocol for the treatment of non-Hodgkin's lymphoma, and the goals of this protocol, namely, to define different prognostic groups within this broad category of patients have largely been achieved. Utilizing a CHOP - high dose methotrexate regimen, the results in lymphoblastic lymphoma without marrow involvement and patients with entirely resected intraabdominal undifferentiated lymphoma or localized disease have been excellent (currently 82% and 90% disease-free survival). There is a suggestion that for some sub-groups of lymphoblastic lymphoma (namely patients with a large mediastinal mass) this protocol may be superior to the widely used LSA_2L_2 protocol. Among the remaining patients the most important prognostic features are soluble interleukin-II level and bone marrow involvement.

Current focus is on high risk patients with undifferentiated lymphomas, defined as all patients other than those with localised disease (stage A) or completely resected disease (stage AR). These patients are being treated on an intensified version of 7704 while a new drug combination of ifosfamide, VP16 and high dose ara-C is evaluated in relapse patients. Because of the evident activity of the relapse protocol, we are planning a new primary protocol incorporating the new drug combination. Several centers have expressed an interest in participating in this study. We should also incorporate molecular analysis of tumors into the new proltocol in an attempt to refine further our ability to sub-categorize undifferentiated lymphomas.

PHS 6040 (Rev. 1/84)

GPO 914-918

Professional Personnel Continued:

David Nelson	Senior Investigator	MET, NCI
David Wagner	Medical Staff Fellow	DCBD, NCI

Major Findings and Future Plans:

A. Prognostic Significance of Soluble Interleukin 2 Levels

We have shown, with members of the Metabolism Branch, that soluble (i.e., circulating) interleukin 2 levels at presentation are the most important prognostic determinant. It is likely that this molecule, which we have shown to be present in Burkitt's lymhoma cells, is an accurate measure of tumor burden, and superior to clinical staging and serum LDH levels in this regard. We are extending this work by developing assays for other lineage specific molecules in serum.

B. Correlation Between Different Disease Sites

We have explored the coincidence of various sites of disease in undifferentiated lymphomas. For example, testicular involvement occurs only in patients with intraabdominal tumor, while jaw involvement is nearly always associated in our patients with diffuse bone marrow involvement and/or other local sites of bone disease. These findings have implications for the biology of the disease (e.g. one can ask what factors govern the particular patterns of spread and begin to explore differences in gene expression or gene rearrangement that correlate with different clinical syndromes), and may sometimes be relevent to therapy (e.g. there is evidence that testicular involvement requires additional local therapy).

C. Evaluation of a New Drug Combination

The new drug combination is highly active in relapsed patients with undifferentiated lymphomas. Among 8 patients so far entered there have been 2 complete responses and 5 partial responses, one patient is too early to assess. A new protocol incorporating this combination into the treatment of high risk patients, as an alternating regimen with the "7704-like" cycles has been designed and will be instituted later this year.

D. Different Sub-Groups of Lymphoblastic Lymphoma

As more patients with lymphoblastic lyphoma accrue on protocol 77-04, it has become apparent that thee are two prognostic groups, which may be designated as "central" (excellent prognosis) and "periphal" (poor prognosis). The validity of this grouping must be confirmed, but it may have important implications, regarding optimal therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 00650-32 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Service Radiation Therapy

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

P.I.:	Eli Glatstein	Senior Investigator	ROB, NCI
Others:	T. Kinsella	Deputy Branch Chief	ROB, NCI
	J. Bader	Senior Investigator	ROB, NCI
	T. DeLaney	Senior Investigator	ROB, NCI
	A. Raubitschek	Senior Investigator	ROB, NCI
	B. Kelly	Chief, Rad. Therapy Tech.	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Therapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	2	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 purpose of this project is to provide expert radiotherapy, consultation, and treatment for patients of the Clinical Cancer Center including patients admitted to services other than the ROB. Support is given to the Medicine Branch, Surgery Branch, Pediatric Branch, NCI/Navy Medical Oncology Branch, Neurosurgical Service, Endocrine Service, and others.

Project Description

Professional Personnel Engaged on the Project:

J. Rowland	Nurse Specialist	CNS, CC
R. Smith	Nurse Specialist	CNS, CC
N. Fox	Nurse Specialist	CNS, CC
L. Campbell	Nurse Specialist	CNS, CC

Methods Employed

Formal and informal consultation with referring physicians and application of radiotherapy where appropriate with x-rays and electrons in accordance with standard radiotherapy practice, as well as modified programs when necessitated by concomitant adjuvant therapies.

Major Findings

Just under 700 patients were seen in formal consultation this year. In addition, between 400 and 500 telephone conversations provided ad hoc advice on treatment for a variety of problems and general information. Approximately 450 patients will be treated this fiscal year with most of these being protocol patients in the Radiation Oncology Branch or on collaborative studies.

Proposed Course

To continue.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 00684-32 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Nonclinical Irradiation Services

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	F. Harrington	Biomed. Engineering Tech.	ROB, NCI
	R. Miller	Radiation Physicist	ROB, NCI
	J. Doolittle	Electronic Technician	ROB, NCI
	P. Foley	Electronic Technician	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	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 Radiation Physics and Computer Automation Section continues to provide radiation physics services, equipment, and advice on experiments involving radiobiology. Cells, tissue cultures, mice, rats, and dogs are irradiated for radiobiology experiments. One current involvement is in I-125 dosimetry related to monoclonal antibody studies.

Considerable efforts have been made and are continuing in assisting both the Radiobiology
Section and Radioimmune Chemistry Section in regard to computerization and automation projects.

Project Description

Objectives: To provide radiation physics expertise and equipment to researchers involved with radiobiological projects.

Methods Employed

Assistance with dosimetric problems and radiation quality assurance continues to be given to radiobiologists in irradiating cells, tissue culture, mice, rats, and dogs usingboth linear accelerators and the Cobalt-60 and 250 kvp x-ray unit. Many devices have been fabricated to both in vitro and in vivo specimen. Extensive assistance is given in the area of automation and computerized data processing.

Major Findings

Cells, tissue cultures and animals are reliably irradiated using the Cobalt-60 unit and the Clinac 20. Basic methodology was developed as well as the linear accelerators.

Significance to Biomedical Research and the Program of the Institute

Radiation physics support is essential to the Radiation Biology Section of the Radiation Oncology Branch. High technology data processing greatly facilitate the evaluation of ongoing research.

Proposed Course

To be continued. Continuing technical support will be provided.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06310-08 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Surgery Versus Radiation Therapy in Treatment of Primary Breast Cancer

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

PI: J.L. Bader Senior Investigator ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Therapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	3	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 purpose of this study is to determine whether a breast-conserving treatment program of limited surgery and definitive radiation offers equivalent local control and survival to mastectomy in patients with early stage breast cancer. After work-up confirms localized disease, patients are randomly assigned to either primary surgery or primary irradiation. Patients treated with mastectomy are offered breast reconstruction. All patients undergo complete axillary node removal; those patients with pathologically positive lymph nodes receive chemotherapy.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Professional Personnel Engaged on the Project:

H. McDonald	Senior Surgeon	SB, NCI
D. Danforth	Senior Investigator	SB, NCI
M. Lippman	Head, Med. Brst. Cancer Sect.	MB, NCI
W. Schain	Clinical Care Consultant	Rehab. Med., CC
N. L. Gerber	Chief, Rehab. Medicine	Rehab. Med., CC
T. d'Angelo	Cancer Nursing Specialist	CNS, CC
M. Lampert	Physical Therapist	Rehab. Med., CC
S. Swain	Senior Staff Fellow	MB, NCI
L. Campbell	Staff Nurse	ROB, NCI

Objectives: If survival and recurrence data obtained with treatment that preserves a cosmetically acceptable breast are comparable to those obtained with radical surgical procedures, such treatment will probably be more acceptable to most women with localized breast cancer. Availability of an effective alternative to mastectomy may encourage woman to seek medical attention with earlier, hence more curable, cancers. The cosmetic and functional results of local treatment will be carefully evaluated. The psychological, sexual and sociological impact of mastectomy vs. lumpectomy and radiation will be noted. Ability to combine aggressive chemotherapy with either local treatment in node positive patients will also be assessed.

Methods Employed

Patients with stage T1-T2, N0-N1, M0 primary untreated breast cancer are candidates for the study. They will be randomized to receive either lumpectomy, axillary dissection and radiation therapy or total mastectomy with axillary node dissection. Patients receiving mastectomy will be offered breast reconstruction. Patients with pathologically positive lymph nodes will receive chemotherapy.

Major Findings

This study has been active for 6 years. Currently 236 patients have been entered, of whom 117 have randomized to mastectomy, and 119 to radiation. Median follow-up is 39 months. No differences have been seen as yet between the two arms in terms of overall recurrence, local recurrence, or survival.

Significance to Biomedical Research and the Program of the Institute

The study is intended to determine whether breast conserving treatment (lumpectomy and radiation therapy) is equivalent to radical surgery as treatment for early stage breast cancer. If this is the case, this

treatment option should be much more acceptable to the majority of women. It is conceivable that the availability of such non-mutilizing treatment would encourage women to seek medical attention sooner, and therefore present with more curable disease.

Proposed Course

The study is ongoing.

Publications

1. Bader, J.L., Lippman, M.E., Swain, S., d'Angelo, T., Danforth, D., McDonald, H., Gerber, N.L., Steinberg, S.: Cosmetic evaluation (CE) following lumpectomy and radiation (XRT) for early breast cancer (BC) is similar with and without adjuvant adriamycin/ cytoxan (AC). Proc. Am. Soc. Clin. Oncol. 6: 62, 1987 (Absract #242).

2. Bader, J., Lippman, M., Swain, S., Danforth, D., McDonald, H., Gerber, L., Steinberg, S., d'Angelo, T., Campbell, L., Findlay, P., Lichter, A., Rosenberg, S., Glatstein, E.: Preliminary report of the NCI early breast cancer (BC) study: A prospective randomized comparison of lumpectomy (L) and radiation (XRT) to mastectomy (M) for stage I-II BC. Proc. Am. Soc. Ther. Radiol. Oncol., Int. J. Radiol. Oncol. Biol. Physics, (in press), Abstract, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06320-08 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Response of Mammalian Cells to Chemotherapy Drugs

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

P.I.	A. Russo	Clinical Associate	ROB,NCI
Others:	J.B. Mitchell	Senior Investigator	ROB,NCI
	W. DeGraff	Biologist	ROB,NCI
	J. Gamson	Biologist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	4	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.)*

Several chemotherapy agents with proven utility, e.g., Anthracylines, Bleomycins, and noble metal derivatives are being studied. The detoxification mechanisms, modification of cellular response by biochemical manipulation of intracellular redox status, and oxygen metabolism, in sensitive and resistant cells are of interest. Deleterious species produced by the antineoplastic drugs and cellular response to these species, as well as thiol compounds, and their metabolic interactions with the drugs, and labile species produced by the drugs are being examined. It has been demonstrated that depletion of cellular glutathione (GSH) by inhibitors of GSH synthesis sensitize cells to Adriamycin and Bleomycin while GSH elevation provides protection. Recently, we have shown that modulation of GSH has a profound effect on Neocarzinostatin biologic activity. Rescue of cells from chemotherapeutic treatment is being studied. The mechanism of transport of these recently synthesized rescue agents and how such transport is subject to intracellular levels of glutathione or glutathione synthesis inhibition is being studied.

GPO 914-918

Project Description

Objective: The objective of this project is to determine the importance of biochemical modulation of selected cellular redox compounds to drug cytotoxicity.

Methods Employed

In vitro cell culture will be exposed to the various agents mentioned above and assayed for cellular reproductive integrity using conventional tissue culture techniques. Both thymic and athymic mice are available to study the in vivo effects of modulation. Standard biochemical assays will be used to access biochemical modulations. Standard molecular biologic techniques are/will be employed. Standard synthetic organic techniques are used.

Major Findings

Cell killing may be enhanced for Adriamycin, Platinum, and Bleomycin after removal of GSH from cells by either of two methods. Protection can be afforded if GSH is elevated by several means. Neocarcinostatin action can be blocked by removal of GSH. Selective differential chemotherapy cytotoxicity was shown for normal vs. tumor cells by GSH modulation. Transport of rescue agents in vitro and in an murine in vivo system appears to be subject to intracellular levels of glutathione as well as levels of glutathione synthesis inhibitor used.

Significance to Biomedical Research and the Program of the Institute

These studies should provide a better understanding of drug-induced resistance and cytotoxicity and are focused toward the potential of selective drug toxicity of tumor versus normal tissue via biochemical manipulation of the cellular redox cycle.

Proposed Course

Dose response curves are being generated for a variety of chemotherapy drugs and the role of intracellular chemoprotective compounds are being studied.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06321-08 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Radiosensitization of Aerated and Hypoxic Mammalian Cells

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

P.I.:	J. B. Mitchell	Senior Investigator	ROB,NCI
Others:	A. Russo	Senior Investigator	ROB,NCI
	J. A. Cook	Staff Fellow	ROB,NCI
	W. Degraff	Biologist	ROB,NCI
	J. Gamson	Biologist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI,NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

There is considerable evidence that the existence of hypoxic cells in human tumors may pose a problem for clinical radiotherapy. The purpose of this project is to study the effects of ionizing radiation delivered at different exposure rates with respect to cell killing, cell cycle status, and cellular redox potential of mammalian cells grown either under aerated or hypoxic conditions. A major portion of this study will be concerned with various means of modulating the cellular redox potential by using drugs that either deplete or elevate cellular glutathione (GSH). Depletion of GSH by buthionine sulfoximine (BSO) enhanced nitroimidazole (2508) sensitization while GSH elevation by oxothiazoidine (OTZ) provided protection of 2508 hypoxic sensitization. Human tumor cell lines were found to be high in cellular GSH and thus less responsive to 2508 sensitization than hamster cell lines. These data may provide explanations for the failure of nitroimidazoles in the clinic. These human tumor cell lines found to be high in GSH can be markedly sensitized to nitroimidazoles by GSH depletion. Preliminary studies have begun using GSH esters to rapidly increase GSH levels and ascertain radiation effects. These esters may be useful in restoring GSH levels of BSO treated cells in an attempt to get back to baseline GSH levels after BSO sensitization.

Project Description

Objective: The objective of the proposed project is to obtain a better understanding of the nature of lesions and processes leading to cell reproductive death and to study the inter-relationships of factors which influence radiosensitivity, with an emphasis on their implications for clinical radiotherapy.

Methods Employed

In vitro cell reproductive integrity will be assayed by the single cell plating techniques for attached cells. Cells will be exposed to radiation, either under aerated or hypoxic conditions. Oxygen enhancement ratios (OER) will be determined. Cellular GSH will be measured by spectrophotometric methods an cellular levels altered by drugs that specifically modulate the GSH cycle.

Major Findings

GSH levels govern SR-2508 hypoxic sensitization and preincubation sensitization to chemotherapy drugs. Human tumor cell lines are high in GSH and do not respond to 2508 at clinically achievable doses. When GSH is depleted in these cells, significant radiosensitization is achieved.

Significance to Biomedical Research and the Program of the Institute

These studies should provide a better understanding of the effects of dose rate/fractionation on the OER. Since there is a good deal of information that indicates that hypoxic cells in tumors represent a problem for radiotherapy, these studies could lead to more efficient methods of sterilizing hypoxic cells. Work with GSH estors may be useful in rapidly restoring GSH levels to depleted tissues after BSO pretreatment.

Proposed Course

Using basic hypoxic cell systems, explore effects of low and elevated levels of GSH on the OER alone in combination with SR-2508.

Publications

1. Mitchell, J.B., Phillips, T.L., DeGraff, W., Carmichael, J., Rajpal, R.K., and Russo, A. The relationship of SR-2508 sensitizer enhancement ratio to cellular glutathione levels in human tumor cell lines. Int. J. Radiat. Oncol. Biol. Phys. 12: 1143-1146, 1986.

2. Phillips, T.L., Mitchell, J.B., DeGraff,W., Russo, A., and Glatstein, E. Variation in sensitizing efficiency for SR 2508 in human cells dependent on glutathione content. Int. J. Radiat. Oncol. Biol. Phys. 12: 1627-1635, 1986.

3. Ashwell, D., Schwartz, R.H., Mitchell, J.B., and Russo, A. Effect of gamma radiation on resting B. Lymphocytes. I. Oxygen-dependent damage to the plasma membrane results in increased permeability and cell enlargement. J. Immul. 136: 3649-3656, 1986.

4. Mitchell, J.B., Gamson, J., Russo, A., Friedman, N., DeGraff, W., Carmichael, J., and Glatstein, E. Chinese hamster pleiotropic multidrug resistant cells are not radioresistant. NCI Monographs (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06329-07 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Clinical Radiation Physics Service

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI
	B. Arora Chin	Radiation Physicist	ROB, NCI
	K. Yeakel-Orr	Dosimetrist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	2.5	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 section continues to provide expert physical and technological support for radiation treatment. This support consists of routine calibration and quality assurance of all radiation equipment and includes special dosimetry studies. computer-assisted treatment planning, and the design and development of special equipment tailored to special clinical needs. Regular checking of dosimetric and technical set-up aspects of radiation treatment will continue.

1. The improvement of the quality assurance program, for the three Varian accelerators (Clinacs 4, 18, and 20) and the Scanditronix Microtron M22 is an ongoing effort.

2. Adaptation of the radiation equipment and special supporting equipment for patient treatment and its implemention is a continuing effort, continually adjusted also to the needs of the ongoing and new clinical research programs.

3. The work on the Microtron is on hold pending its repair and upgrading.

4. The computer programs for clinical radiation treatment planning are being further extended in all three subfields: external beam, point-source, intracavity line-source, and interstitial radioactive seeds radiation fields. Support of intracranial implants continues to be of particular interest in this regard.

5. Extension of the clinical usefulness of the VAX-750 Computer System is continuing. A MicroVAX system and several Macintosh systems have been added to improve the flexibility and to provide better support for ongoing research.

6. Supporting patient treatment and evaluation of clinical research.

Project Description

Personnel:

J. Pochobradsky	Computer Specialist	ROB, NCI
E. Lamoreaux	Computer Specialist	ROB, NCI
J. Doolittle	Electronics Specialist	ROB, NCI
P. Foley	Electronics Specialist	ROB, NCI

Objectives: To ensure high quality physics support for radiotherapy.

Methods Employed

The locally developed highly efficient system for daily and periodic quality assurance is continually used for monitoring the performance of three linear accelerators, the Microtron, the simulator, and the CT scanner. Special mechanical supports and measuring devices are used to quantify the position of patients and to improve the reproducibility of daily patient set-ups. The data acquisition for treatment planning have been simplified and improved. Computer-assisted treatment planning has been extended. Considerable efforts have been invested in the dosimetry of intraoperative, total-body, and total-skin radiotherapy. Pending the restoration of the microtron , the intraoperative work is continuing on the Clinic-20. Appropriate adaptive work has been performed. The CT scanner provides vastly improved quantitative data and superior image resolution, allowing thinner slices and more readily accommodates patients in the treatment position. This, in turn, allows much higher treatment planning.

The Section continues to provide non-routine in vivo patient dosimetry by means of thermoluminescent dosimeters and diodes. Such ad hoc measurements are usually concerned with doses to sensitive organs and sometimes crucial to the continuation of a treatment technique.

Major Findings

Beam monitoring locally developed and other quality assurance support jigs enable daily monitoring of output, beam flatness, symmetry, and alignment of light field and x-ray fields for all three linear accelerators. The method allows simple documentation of performance. This close surveillance of beam quality has proved to be a vital factor in treatment reliability and quality. Our system continues to impress visitors. The dosimetry of photon beam total-body irradiation, as well as that of total-skin electron beam irradiation for mycosis fungoides, requires further attention especially in improving the treatment of hands and feet.

The most important contribution in computer-assisted treatment planning is the availability of routine interactive optimization and routine multi-slice imaging of dose distributions superimposed on CT scans. An important improvement is the capability to image the effects of irregular shielding blocks which is of essential interest in the treatment of soft-tissue sarcomas and cancers of the esophagus.

The use of locally designed and developed equipment and methodology continues to be a major factor in quality control of equipment, methodology and treatment documentation. This is especially important in view of the generally highly complex clinical studies in this Branch.

The development of a mechanical back projection system for reconstruction of the positions of objects in the body has been highly successful.

Over the reporting period, the Section has been severely handicapped by understaffing. Over one-half year the regular complement of four physicists was down to two.

Significance to Biomedical Research and the Program of the Institute

The improvements in quality assurance, patient positioning, and treatment planning are essential as a basis for optimal patient treatment and for meaningful evaluation of treatment protocol studies. The CT scanner is now the principal source of patient data for treatment planning.

Proposed Course

1. Continuation of adaptation of the computer programs to the new radiation machines and full usage of the VAX system.

2. Special attention to the quality assurance aspects of the Microtron, currently under installation.

3. Introduction of integration of NMR imaging into treatment planning purposes.

Publications

1. Miller, R.W., van de Geijn, J.: Modification of the fault logic circuit of a high-energy linear accelerator to accommodate selectively coded, large-field wedges. Medical Physics 14:262-264, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06330-07 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Radiation Field Modeling

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	J. Pochobradsky	Computer Specialist	ROB, NCI
	E. Lamoreaux	Computer Specialist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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.)*

This is fundamentally ongoing research and development. The capability to calculate the distribution of absorbed dose produced by photon beams and electron beams of the most general characteristics is of vital importance in radiotherapy. Conceptually, this radiation field model takes as a basis the empirical distributions along three mutually perpendicular reference lines in a "master field." Mathematical expressions describe the effect of variations of field size, depth and focal distance. This concept is applied to the beam-modifying devices as well. The approach is attractive from a theoretical as well as a practical point of view. The present investigations include the generalization of the treatment of the influence of inhomogeneities. New concepts in the description of the along-the-ray distribution as well as the field size dependence of the beam profile have been developed and are being implemented. A completely new approach to electron field modeling is well underway and extremely promising. Of special interest are the implications of the large number of electron energies and the need for flexible application of different energies and field shapes in combination with photon fields. For the high energy photon

beams, the Net Fractional Dose Concept (NFD) has been extended (ENFD). The ENFD formalism includes the description of the influence of inhomogeneities, such as lung tissues. It includes correction for secondary electron transport.

Project Description

Objectives: To extend unified calculative models for the description of absorbed dose produced by beams of ionizing radiation, including photon beams as well as electron beams, as a basis for computer-assisted treatment planning, with special attention to high energy x-ray and electrons.

Methods Employed

1. The variation of relative absorbed dose along the central ray with depth, field size, and source surface distance (SSD) has been studied using published and locally measured data. Mathematical representations have been established for a range of energies now covering 60 Co to 20 MV x-rays. These formulations have been extended to inhomogeneities. The concept of lateral electron build up has been introduced to account for the lateral range of high energy secondary electrons.

2. The variation of the relative absorbed dose across the beam has been studied as a function of field size, depth and SSD for many radiation qualities for photons, electrons, and neutrons. Mathematical representations for these variations have been established. A new concept (the collimator function) is being implemented, for the generalized description of beam profiles.

3. Special attention has been paid to verification of the model for the local radiation machines. This work is continuing and will be extended to the 6 MV and 21 MV x-ray beams of the Microtron.

4. New concepts are being explored in electron beam modeling, including the concept of differential electron scatter functions, based on separation of the primary electron component and the various contaminations in clinical electron beams.

Major Findings

In x-ray dose field modeling, the description of electron transport correction has proven to be highly significant especially in high energy x-ray treatment with small fields in the thorax. Preliminary results for electron beams are most promising. The new electron beam model is both simple and more accurate than any other current approach. All of these results are being incorporated in a clinical treatment planning system.

Significance to Biomedical Research and the Program of the Institute

The range of validity of the dose field model determines the potential range of applicability of the clinical treatment planning program. In turn, the latter determines the degree of refinement in radiation treatment that can be scientifically documented.

Proposed Course

Continuation, with the emphasis of inhomogeneities in photon and electron beams. In regard to electron beams, the influence of oblique incidence, non-standard distances between electron applicators, and patient surface need further attention, especially in view of moving electron beam.

Publications

1. van de Geijn, J., Miller, R.W.: A new description of the photon beam peak-depth profile as a function of field size. Medical Physics 13:904-907, 1986.

2. van de Geijn, J.: The extended net fractional depth dose: Correction for inhomogeneities, including effects of electron transport in photon beam dose calculation. Medical Physics 14:84-92, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06331-07 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Computer-Assisted 3-D Treatment Planning

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	B. Chin Arora	Clinical Physicist	ROB, NCI
	E. Lamoreaux	Computer Specialist	ROB, NCI
	J. Pochobradsky	Computer Specialist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Physics and Computer Automation 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.)*

The purpose of this continuing project is the development and clinical implementation of a generalized system for external beam treatment planning. It will enable the optimum utilization of existing treatment facilities. The system is based on a generalized 3-D dose field model which covers photon and electrons as well as neutron beams. The computer program and most of its clinical implementation was completed for the photon and electron fields available from the local Clinac 4, Clinac 8 and Clinac 20 linear accelerators. Work on the implementation of the Microtron with its 2 photon energies and 9 electron energies and some unusual technical options is currently on hold awaiting extensive modification of the microtron. A new generalized description of the cross-beam dose profile has been developed and published, as has an important extension of the "Net Fractional Depth Dose," which now comprises a description of the influence of electron transport. Continuing is optimization of most of our computer programs on the VAX-11/750 system. Work on implementation of a new locally developed electron beam model of great simplicity and accuracy is continuing. Progress of this work is, as always, complicated by the need for continuing reliable routine support for the clinical treatment.

Project Description

Personnel:

K. Yeakel-Orr	Dosimetrist	ROB, NCI

Objective: To develop and implement a generalized system for computer-assisted radiation treatment simulation.

Methods Employed

The dose field model for external beam dosimetry, originally developed elsewhere by the present principal investigator, is continually being further developed and experimentally tested. The theoretical model covers irregularly shaped beams as well as irregularly shaped shielding blocks. Major work has been done on the improvement of the correction for inhomogeneities, such as lung tissues, and electron beam dosimetry. Considerable improvement has been achieved by the extension of the Net Fractional Depth Dose Concept, which now covers inhomogeneity correction, including correction for secondary electron transport. The facility enabling the computation of display of dose distributions in planes perpendicular to the respective beam axes is being extended.

Major Findings

The system, although continuing to be further expanded, is in routine use for clinical treatment planning. In comparison to other systems, operational as well as under development, it offers very high quality, high speed computation and display of complete dose distributions in multiple slices, superimposed on CT images, for practically any clinical treatment planning problem. Several modes of display are available. The facility has a major impact on the conceptual understanding of the spatial aspect of radiation treatment dosimetry, as well as its application in clinical research programs. The capabilities of the programs for interstitial treatment planning are of vital importance to the brain implant study.

The Radiation Oncology Branch is continually engaged in innovative clinical studies. Detailed and accurate treatment planning is of essential concern, more so than in other oncology institutes. The quality, versatility and flexibility of our treatment planning system has proven to be indispensible to this research.

Significance to Biomedical Research and the Program of the Institute

The convenient interactive manipulation of the key beam parameters in combination with fast response is highly valuable in the complicated dosimetry problems encountered in special protocol studies. The facility is also highly effective in the Resident's Training Program. Especially versatile 3-D imaging currently being implemented will greatly improve knowledge of dose distribution vs. anatomy.

Proposed Course

1. Further development of the especially new imaging techniques.

2. Extension of the new locally developed electron beam model to cover inhomogeneity.

3. Establishment of a "Slave Monitor System" to enable the display and limited modification of treatment plans during the daily patient conferences; this is still being delayed by budgeting problems.

4. Extension of the capabilities to compute and display dose distributions in sagittal, coronal and Beam's Eye View sections of the patient on an interactive basis.

5. Development and implementation of digital x-ray imaging in conjunction with computerized treatment planning; this has also been delayed by both budgeting and personnel problems.

6. Development of digital x-ray imaging in the realm of comparison of simulator (set-up) films and treatment machine port films.

Publications

1. van de Geijn, J., Miller, R.W.: A new description of the photon beam peak-depth profile as a function of field size. Medical Physics 13:904-907, 1986.

2. McKenna, W.G., Yeakel, K., Klink, A., Fraass, B.A., van de Geijn, J., Glatstein, E., Lichter, A.S. Is Correction for Lung Density in Radiotherapy Treatment Planning Necessary? Int. J. Radiat. Oncol. Biol. Phys. 13:273-278, 1987.

3. van de Geijn, J.: The extended net fractional depth dose: Correction for inhomogeneities, including effects of electron transport in photon beam dose calculation. Medical Physics 14:84-92, 1987.

4. Miller, R.W., van de Geijn, J.: Modification of the fault logic circuit of a high-energy linear accelerator to accommodate selectively coded, large-field wedges. Medical Physics 14:262-264, 1987.

5. van de Geijn, J., Chin, B., Pochobradsky, J., Creecy, R.H., Miller, R.W.: A New Model for Clinical Electron Beam Dosimetry. Medical Physics, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06333-07 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Dosimetry of Total Skin Electron Irradiation

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Other:	R. Miller	Radiation Physicist	ROB, NCI
	B. Chin Arora	Radiation Physicist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI
	R. Morton	Radiation Physicist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A detailed study has been made previously of the dosimetry of total skin electron irradiation. This study has quantified and improved the whole skin treatments received by patients with mycosis fungoides (MF). The treatment technique has now been updated and implemented on the new Clinac 20 linear accelerator.

A study has been started to selectively and locally modify the fluence rate to allow for the locally higher dose rates to the hands and feet, which now require cumbersome shielding over part of the treatment course.

Total skin electron radiation is given routinely on the Clinac 20. The project directed at modification of electron fluence distribution by means of a grid technique has shown promising initial results. Practical application needs some further work. A paper is in preparation. In the meantime, the "gloves-and-boots" (protection of hands and feet) have been made considerably less uncomfortable and more effective, improving the conditions for consistent treatment.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objective: Quantify and improve whole-skin treatments received by patients with mycosis fungoides.

Methods Employed

A grid method is under development enabling local modification of beam intensity without changing the energy. The method has been found to be feasible. Practical details are being worked-out. Conventional protection of extremities and general patient comfort have been improved considerably.

Major Findings

The currently available electron energy used for total skin irradiation is felt to be marginally useful through a clinical standpoint. Current research is aimed at boosting the effective electron energy for this mode.

Significance to Biomedical Research and the Program of the Institute

This work improves treatment for mycosis fungoides with the whole-skin irradiation technique. Modification of the beam intensity at the hands and feet of MF patients will greatly reduce their discomfort during treatment. Modification of hand support protection and feet protection has improved treatment reproducibility.

Proposed Course

Work toward improving the dose distribution is continuing.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06349-06 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Relationship of Cellular Redox State and Thermotolerance

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

P.I.:	A. Russo	Clinical Associate	ROB,NCI
Others:	J. B. Mitchell	Senior Investigator	ROB,NCI
	W. DeGraff	Biologist	ROB,NCI
	J. Gamson	Biologist	ROB,NCI
	N. Friedman	Biologist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	5	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.)*

Hyperthermia is currently being evaluated as a potential cancer treatment modality. The mechanism(s) of hyperthermia killing and the induction of thermal resistance (thermotolerance) are not known. We will examine the role of the cellular reduction potential during and after heating to determine its role or alteration during thermal stress. This will be accomplished by using drugs which either bind GSH or prevent its synthesis. There appears to be a relationship between the synthesis of heat shock proteins and the induction of heat resistance. The effect of thiol modulation will be studied in the context of heat shock proteins. Recently, several compounds have been introduced which elevate cellular GSH. These compounds will be synthesized and evaluated in regard to thermal response. Continued effort to inter-relate oxidative stress and the biochemical induction of genetic materials center around GSH metabolism. There is also interest in the role that GSH has on maintaining the integrity of the membrane. Heat sensitizers, such as polyamines will be studied to determine their impact on the cellular redox systems as reflected by GSH levels. The effect of hyperthermia stress on redox status is continuing to be investigated. Particularly attention is being directed to dissecting out the components of the heat stress response and oxidative stress related enzymes.

Project Description

Objective: To determine how the cellular redox state is altered during thermal stress.

Methods Employed

In vitro cell cultures will be exposed to heat and assayed for reproductive integrity using conventional tissue culture techniques and assayed for various biochemical compounds important in maintaining the cellular redox state.

Major Finding

There is a relationship in elevation glutathione and the induction of thermotolerance. Thermotolerance may be prevented by lowering cellular GSH or preventing its synthesis and an alteration in the extent of heat shock proteins synthesized. Polyamines, heat sensitizers, markedly deplete cellular GSH levels.

Significance to Biomedical Research and the Program of the Institute

These studies should provide a better understanding of how heat kills cells, which might provide a clearer means for clinical utilization of hyperthermia as a treatment modality.

Proposed Course

Continue studying the relationship of glutathione (a cellular reducing compound) and thermotolerance.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06351-05 RO

PERIOD COVERED

October 1, 1987 to September 30, 1987

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

Response of Mammalian Cells to Halogenated Pyrimidines

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

P.I.:	J.B. Mitchell	Senior Investigator	ROB,NCI
	J.A. Cook	Staff Fellow	ROB,NCI
Others:	A. Russo	Senior Investigator	ROB,NCI
	W. DeGraff	Biologist	ROB,NCI
	J. Gamson	Biologist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI,NIH, Behtesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	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.)*

When certain halogenated pyrimidines such as bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) are incorporated into cellular DNA, the cells become more sensitive to ionizing radiation. This observation has led to several clinical studies over the years and recently at the NCI to evaluate whether selective sensitization of tumors could be achieved by IdUrd infusion followed by radiation. An important question arises in these studies regarding whether or not the drug actually is incorporated into cells. This study proposes to obtain information regarding this question by using: a) a IdUrd monoclonal antibody and HPLC assays to actually quantitate the amount of IdUrd in tumor compared to normal tissue; b) IdUrd monoclonal antibody to sort out S phase cells by flow cytometry and determine labelling index of human tumors; and c) evaluation of the possible potentiation of chemotherapy drugs by IdUrd incorporation. Further studies have questioned the role of low dose rate irradiation with halogenated pyrimidines.

Project Description

Objectives: To quantitate the amount of IdUrd in tumor vs. normal tissue by flow cytometry. With these techniques, optimal timing schedules of incorporation for maximum differential radiosensitizers will be determined. To determine if halogenated pyrimidine incorporation enhances chemotherapy response.

Methods Employed

A monoclonal antibody for IdUrd and HPLC assays will be used to quantitate incorporation of IdUrd in tissues. Standard cell survival techniques have been used for other mammalian cell systems.

Major Findings

Positive identification of cells in tumor and normal tissue that had incorporated BrdUrd and IdUrd has been made using the monoclonal staining technique. These studies should provide a better understanding as to quantities of IdUrd required to radiosensitize cells from tumor and normal tissue in a clinical setting. Low dose rate radiation is potentiated by IdUrd incorporation into cellular DNA. This observation has major clinical implications. Additionally, incorporated IdUrd enhances certain chemotherapy cytotoxicity, particularly for drug resistant cells.

Significance to Biomedical Research and the Program of the Institute

These studies should provide a better understanding as to quantities and timing of IdUrd required to radiosensitize cells from tumor and normal tissue in a clinical setting and the possible efficacy of combining implant therapy to the use of halogenated pyrimidines.

Proposed Course

Continue work on cellular quantitations of IdUrd. Evaluate cell survival of other mammalian cells to halogenated purines and work out timing of incorporation for maximum differential sensitization.

Publications

1. Mitchell, J.B., Russo, A., Kinsella, T.J., and Glatstein E. The use of non-hypoxic cell sensitizers in radiobiology and radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 12: 1513-1518, 1986.

2. Russo, A., DeGraff, W., Kinsella, T.J., Gamson, J., Glatstein, E., and Mitchell, J.B. Potentiation of chemotherapy cytotoxicity following iododeoxyuridine incorporation in chinese hamster cells. Int. J. Radiat. Oncol. Biol. Phys. 12: 1371-1374, 1986.

3. Kinsella, T.J., Dobson, P.P., Mitchell, J.B., and Fornace, A.J. Enhancement of x ray induced DNA damage by pre-treatment with halogenated pyrimidine analogs. Int. J. Radiat. Oncol. Biol. Phys. 13: 733-739, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06352-05 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Relaxation Agents for NMR Diagnostic Imaging

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

P.I.:	O. A. Gansow	Senior Investigator	ROB, NCI
Others:	M. W. Brechbiel	Chemist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Inorganic and Radioimmune Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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.)*

Nuclear Magnetic Resonance (NMR) imaging is a most powerful method for the non-invasive diagnosis of disease. A fundamental limitation of the technique derives from the fact that images are constructed from T1 relaxation time measurements of protons in the various biological "compartments". If T1 values for differing soft tissue types are similar, the type will not, in general, be resolvable in the images. A potential method for improving this situation is the development of relaxation agents which specifically alter T1 relaxation rates in tissues where they may be concentrated.

A study of concentration dependence of T1 relaxation by various metal chelates and organic nitroxyl radicals has been prepared. Based on these studies, the metal chelates appear to be more efficient.

We have recently shown that metal chelates are useful as an NMR contrast agent in myelography and cisternography. We have prepared a new and superior contrast agent, Gadolinium (Gd)DOTA.

This year we report numerous applications of contrast agents for in vivo MRI imaging.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Professional Personnel Engaged on the Project:

J. Frank	Clinical Associate	DR, CC

Objectives: We proposed to construct paramagnetic molecules that localize in certain biological compartments in order to reduce T1 relaxation times of water in the area. We plan to attach paramagnetic metal chelates to proteins found to localize where desired in the body. The idea is that since paramagnetics alter local T1 values, by concentrating them in differing tissue types, we could induce resolution in NMR images. For example, paramagnetic labels attached to blood proteins, which circulate freely blood, thus allowing imaging of cardiac function and blood flow. A second example, would be to label tumor associated monoclonal antibodies. In recent work done in this section, it has proven possible to localize radioisotopes attached to antibodies in tumors by using metal chelates.

Methods Employed

Bifunctional metal chelates capable of securely binding paramagnetic metals like iron, chromium or gadolinium have been prepared and attached to the proteins described above. The effect of these paramagnetic relaxation agents on T1 values have been measured by conventional inversion, recovery methods. The chelate, Gd (DOTA) is being tested as an MRI contrast agent (CA) by examination of relaxation times in aquem media and in tissues excised from appropriate animals. In vivo studies of the MRI of CA topes model are also in progress.

Major Findings

Studies have now shown that paramagnetic chelates may be attached to antibodies or albumin without affecting the biological properties of the proteins. Results of T1 studies show that many paramagnets must be attached to one protein to have an effect in vivo.

Use of Gd(DTPA) as an NMR contrast agent has demonstrated that MRI myelography and cisternography may be practically useful in the clinic.

The T1 measurements required to determine whether labeled paramagnetic proteins could be of use in vivo have been done. Synthetic chemical procedures necessary for attachment of many chelates to protein were developed.

We have prepared kilogram quantities of a new contrast agent Gd(DOTA) for use in humans. A bifunctional DOTA chelate has been prepared.

1. DiChiro, G., Girton, M., Frank, J., Dietz, M.J., Gansow, O.A., Wright, D.C. and Dwyer, A.J.: Cerebiospinal Rhinorrhea: Depiction with MR Cisternography in Dogs. Radiology 160, 221-222, 1986.

2. Frank, J.A., Dwyer, A.J., Girton, M., Knop, R.H., Sank, U.J., Gansow, O.A., Magerstast, M., Brechbiel, M., Doppman, J.C.: Opening of Blood-Oculor Barrier Demonstrated by Contrast-Enhanced MR Imaging. J. Computer Asst. Tomography, 10: 912-916, 1986.

3. Magerstadt, M., Gansow, O.A., Brechbiel, M.W., Colcher, D., Baltzer, L., Knop, R.H., Girton, M., and Naegele, M.: Gd(DOTA): An alternative to Gd(DTPA) or a $T_{1/2}$ Relaxation agent for NMR Imaging or Spectroscopy. Magn. Res. in Med., 3: 808-812, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06353-05 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Metal Chelate Conjugated Monoclonal Antibodies for Tumor Diagnosis and Therapy

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

PI:	O. A. Gansow	Senior Investigator	ROB, NCI
Others:	S. M. Mirzadeh	Expert	ROB, NCI
	M. Brechbiel	Chemist	ROB, NCI

COOPERATING UNITS *(if any)*

Johns Hopkins Medical School, Baltimore, MD (M. Strand); Argonne National Laboratory, Argonne, IL (R. W. Atcher)

LAB/BRANCH

Radiation Oncology Branch

SECTION

Inorganic and Radioimmune Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	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.)*

Tumor-associated monoclonal antibodies are potential therapeutic agents as selective carriers of cytotoxic agents to malignant cells. We are testing this hypothesis in several animal model systems: one is a tumor virus induced leukemia of mice, another is human tumor xenographs in nude athymic mice.

The various cytocidal agents being employed are radioisotopes. Their relative therapeutic efficacy when conjugated to antibodies is being assayed and compared to that of monoclonal antibodies alone. The isotopes now being used include the highly tumoricidal alpha emitting parent radioisotopes Pb-212 or Bi-212, as well as the beta particle emmiter, Y-90. The syntheses of different chelates and radiochemical separations required for these objectives are being devised and reduced to clinical practice. Results from isotopic therapy are being compared with those obtained by use of antibody conjugated toxins or drugs with respect to tumor growth, regression or cure.

These studies will provide for human medicine a basis for design of rational therapy of malignancies by selectively targeting cytocidal agents to tumors as well as metastases.

New chelates for use in this project have been synthesized and used and have thus far proven useful for radiobiology studies of cell killing with alpha particle labeled antibody and for imaging of tumors in mice.

Patient protocols for imaging of human tumors by use of In-111 labeled monoclonal antibody B72.3 have been initiated.

Project Description

Professional Personnel Engaged on the Project:

D. Colcher	Senior Investigator	LCMB, NCI
T. Waldmann	Chief	MET, NCI
R. Kozak	Staff Fellow	MET, NCI

Objectives: The specific goal of these studies is to investigate in vitro and in animal tumor models the therapeutic efficacy of radionuclides attached to tumor associated monoclonal antibodies. These studies encompass the synthesis of new bifunctional chelates designed for therapy employing a variety of radioisotopes and radiation types.

Methods Employed

Methods for covalently conjugating metal isotopes in bifunctional chelates to monoclonal antibodies are being devised and developed. The inorganic chemistry of new complexing agents for metal isotopes thought to be useful in tumor diagnosis or therapy is being explored. The objectives of the research must thereby of necessity include: (a) the synthesis and characterization of new bifunctional chelates and their metal complexes, both before and after protein conjugation; (b) the evaluation of currently available chelates for use as carriers of isotopes familiar in clinical environments (e.g., Tc-99M) and of less common, but potentially serviceable radionuclides (e.g. Ga-68, In-111, Pb-212, Bi-212, Y-90), (c) the development of chemical procedures (protocols) for routine and reproducible preparations of rigorously stable radiometal chelate conjugated monoclonal antibodies which retain their inherent biological specificity and activity and (d) the use of animal models for investigating the stability in vivo of metal labeled antibodies.

Major Findings

Some of the problems which have been addressed include: (1) the incorporation of Bi-212 into chelates attached to antibody; and (2) the evaluation of three DTPA chelates and an EDTA chelate for use in antibody modification.

1. A new generator for the Pb-212, Bi-212 radionuclides is now being provided by Argonne National Laboratory and is in use to label antibodies for radiobiology studies.

2. Four chelates have been used for antibody labeling; the DTPA dianhydride, the DTPA isobutycarboxycarbonic anhydride, the para-isothiocyanatobenzyl derivatives of DTPA and EDTA. Methods for

labeling antibody with In-111 have been developed. It was found that the benzyl DTPA chelate gave superior tumor images when used to label antibody BF2.3 with radioindium.

3. A study of the relative cytotoxicity of x-particle linked antibody vs. conventional gamma radiation (CS-137 beam) was performed. The radioimmunotherapy was shown to be superior.

4. Chemical methods for labeling antibodies with Y-90 have been developed.

5. New chelates for Pb-212 have been synthesized.

Significance to Biomedical Research and the Program of the Institute

The ability to attach metals to antibodies is significant for several reasons. It enables one to diagnose and detect cancer using radioactive metals in nuclear medicine tests, or using paramagnetic metals to enhance nuclear magnetic resonance images. The ability to attach particle emitters to antibodies opens up site specific therapy using a variety of radioactive metals which can be selected to maximize cell killing while sparing normal tissue. Finally, it appears that the bifunctional chelates currently being investigated have little effect on the viability and specificity of the antibodies, thus preserving their function.

Proposed Course

The effects of the number of chelates attached to the antibodies and the other conditions of preparation on the activity of the antibodies both in vitro and in vivo will be examined. Next, the conditions under which the metals are incorporated into the chelates will be examined for their effects on biological activity both in vitro and in vivo.

The radiobiology studies now underway will be expanded to test a number of human cell lines. Those cell lines adequately treatable will be proposed for study in animal models.

Protocols for diagnosing colon cancer and treatment of T-cell lymphoma in humans are being implemented in the clinic.

Publications

1) Kozak, R.W., Waldmann, T.A., Atcher, R.W., Gansow, O.A.: Radionulcide-conjugated monoclonal antibodies: a synthesis of immunology inorganic chemistry and nuclear science. Trends in Biotech. 4, 259, 1987.

2) Biechbiel, M.W., Gansow, O.A., Atcher, R.W., Scholm, J., Estebam, J., Simpson, D.E., and Colcher, D.: Synthesis of 1-C-(p-Isothiocyanatobenzol) Derivatives of DTPA and EDTA. Inorg. Chem. 25, 2772-2781, 1986.

3) Esteban, J., Schlom, J., Gansow, O.A., Atcher, R.W., Biechbiel, M.W., Simpson, D.E., and Colcher, D.: New Methods for the chelation of Indium-111 to monoclonal antibody. J. Nucl. Med. 28, 861-867, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06354-05 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Iron-57 and H-2 Nuclear Magnetic Resonance: New Tools for Biomedical Research

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

PI: O. A. Gansow Senior Investigator ROB, NCI

COOPERATING UNITS *(if any)*

Colorado State University, Fort Collins, CO (S. S. Strauss).

LAB/BRANCH

Radiation Oncology Branch

SECTION

Inorganic and Radioimmune Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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.)*

We are developing iron-57 and deuterium Nuclear Magnetic Resonance (NMR) as an experimental method for use in the Biomedical Sciences. Many compounds found in the blood contain iron cores. Among these are the cytochromes, hemoglobin, or ferridoxin. Few chemical methods may be used to directly study the central metal atom environments of these proteins. Iron-57 NMR is being developed for that purpose.

Some time ago, we reported the utility of iron-57 NMR for study of relaxation times and chemical shifts of some iron compounds related to biological entities. We determined thus the basic parameters needed for further study of irom NMR.

Our current efforts to extend this project to include an examination of the field dependent H-2 NMR of iron porphyrins as a companion method to iron NMR have resulted in measurements of anisotropies on iron porphyrins partially oriented in a magnetic field.

Project Description

Professional Personnel Engaged on the Project:

E. Becker	Section Head	LCP, NIADDK

Objectives: We plan to measure the iron and deuterium NMR signals from a number of biological, inorganic and organometallic compounds. The goal of the project is to define the experimental conditions and parameters necessary for the direct detection of iron and deuterium NMR in order to explore its utility for studies of biological processes.

To develop the method, a knowledge of two physical parameters of iron NMR signals must be obtained. They are chemical shift values and T1 relaxation times. By observing resonances of inorganic and organometallic model compounds, it is possible to define the chemical shift scale for the iron nucleus. These data serve to define the resolution of the method. Similarly, by measuring T1 relaxation times of these compounds, we will learn how to optimize chemical environments and experimental conditions required to detect iron resonance. With that information in hand, a rational selection of biological problems amenable to study by this method can be effective.

Methods Employed

A specially constructed NMR probe for observing iron NMR has been built and used as described in our recent publication. Enriched iron-57 proteins such as myoglobin have been synthesized. A new NMR probe for use with biological molecules has been constructed and tested successfully. Probes for H-2 NMR have also been employed to measure H-2 NMR of porphyrins.

Major Findings

We have accomplished the first direct detection of iron-57 NMR in biological molecules. We have undertaken the first systematic study of iron-57 chemical shift values. Initial results indicate a range of > 5000 ppm. A study of iron-57 relaxation times showed the advantage of the experiment being done at high magnetic fields.

These same types of porphyrins were studied by H-2 name. We found that we could measure magnetic anisotropies by this method and determined that iron octaethyl porphrin and iron octaethylcorrin have similar aial magnetic anisotropies despite not having congruent electronic structures.

Significance to Biomedical Research and the Program of the Institute

Our studies have shown that iron-57 and H-2 are new and valuable methods for study of biological compounds. We have shown that ^{57}Fe NMR of proteins can be observed. We have developed a simple method for measuring magnetic anisotrophies of the iron porphyrins.

Publications

1. Strauss, S.S.; Long, K.M.; Magerstadt, M.; Gansow, O.A.: Iron Porphyrin and Hydroporphyrin Magnetic Anisotropies Derived from High Field ^{2}H NMR Spectra. Inorg. Chem., 26 1185-1187, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06356-04 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Treatment of Malignant Brain Tumors with Interstitial Radiotherapy

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

P.I.:	T. Kinsella	Senior Investigator	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	K. Orr	Dosimetrist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Therapy Section and Physics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

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

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

Median survival of high-grade gliomas remains less than a year despite multi-modality treatment. Cure is considered anecdotal. These tumors usually have extended beyond the limits of a complete surgical resection and a dose of conventional external beam radiotherapy has been limited by surrounding brain tolerance. We believe that we can achieve a higher radiation dose to the tumor by placing radioactive seeds of Iodine 125 directly into the tumor bed, with a sharp fall-off of radiation to the surrounding normal brain. Hopefully this will achieve a much better therapeutic ratio, especially when delivered at low dose rates.

Project Description

Professional Personnel Engaged on Project:

D.C. Wright	Senior Investigator	SN, NINCDS

Objectives: To develop a technique of interstitial implantation of intracranial tumors; to determine the acute effects and complications of such treatment; to explore the efficacy of such therapy; and to develop patient selection guidelines for future applications.

Methods Employed

Patients with primarily untreated high-grade gliomas of less than 5 cm diameter and enhancing lesions of CT scan receive approximately 4000 rads of external beam radiotherapy prior to implantation. Implants with similar tumors recurrent after prior standard treatment receive implant only. Using a Brown Robert Wells sterotactic frame and a suctomized template device which we have made ourselves, silastic catherters loaded with radioactive seed of Iodine 125 are sterotactically positioned in the tumor. Catheters are then anchored to the dura and the bone defect is closed.

Major Findings

Fourteen patients have been enrolled. The four patients with recurrent tumors received implants and all died in less than one year. Ten patients with no previous treatment have been entered on the protocol. Four have died in less than a year and the other six are still alive up to twenty months from the beginning of treatment.

A technique for the sterotactic placement of multiple catheters containing multiple radioactive sources has been devised. It can be used to implant a tumor in any cranial site, exluding the posterior fossa, which is simply inaccessible. It can be adapted to a variety of isotopes and to a variety of tumor configurations.

Significance

This study helps to provide information on dose and dose rate effects on both tumor and normal brain.

Proposed Course

This study is now being modified to include the radiosensitizer IUdR, which is capable of sensitizing cells to even low dose rate isotope exposures.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06357-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Clinical Studies on Intraoperative Radiation Therapy

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

PI:	T. J. Kinsella	Deputy Branch Chief	ROB, NCI
Others:	E. Glatstein	Chief	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Therapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	10	

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 Radiation Oncology and Surgery Branches of the National Cancer Institute have been involved in prospective randomized studies evaluating the potential role of intraoperative radiotherapy in three major disease sites including resectable and unresectable carcinomas of the pancreas, resectable carcinomas of the stomach, and resectable retroperitoneal sarcomas. We have also been involved in single arm pilot trials involving intraoperative therapy in selected patients with locally advanced tumors not felt likely to be cured by standard therapy such as locally advanced lung carcinomas. To date, 105 patients have been treated with experimental intraoperative radiation therapy on these various protocols and there are an additional 65 other patients being followed as control patients on the various randomized prospective trials. The randomized study in patients with resectable pancreatic carcinoma demonstrates a significant improvement in local control and overall survival. Preliminary evaluation of patients with resectable gastric carcinoma also documents an improvement in local control and an increase in survival, although not statistically significant. In patients with unresectable pancreatic carcinoma and in large resectable retroperitoneal sarcomas, there appears to be no benefit to the use of intraoperative radiation therapy. The trials on pancreas carcinoma and retroperitoneal sarcomas have been closed. Patients are still being accrued for the pilot studies, as well as for the resectable gastric carcinoma study.

GPO 914-918

Project Description

Professional Personnel Engaged on the Project:

W. Sindelar	Senior Investigator	SB, NCI
H. Pass	Senior Investigator	SB, NCI
R. Smith	Cancer Nursing Specialist	CNS, CC
M. Maher	Cancer Nursing Specialist	CNS, CC

Objectives: These are Phase I & II studies assessing the role of intraoperative therapy as an adjunct to surgical resection in various primary tumor sites including pancreas, stomach and retroperitoneum where local failure following surgery alone is unacceptably high. Additional pilot studies are on-going to determine the role of intraoperative radiation therapy for tumors with a high risk of local recurrence. A dedicated intraoperative suite is available for these studies in the Radiation Oncology Branch.

Methods Employed

The patients are considered for entry on the randomized prospective trials with combined surgical resection and intraoperative radiation therapy that have specific malignant lesions within the abdomen and retroperitoneum and no evidence of metastatic spread. In general, the control arm of these studies receives resection with post-operative conventionally fractionated external beam radiation. All patients receiving intraoperative radiation therapy are given misonidazole, a known radiation sensitizer of hypoxic cells at a dose of 3.5 gm/m^2. The patients are closely followed to assess local toxicity and patterns of recurrence.

Major Findings

To date, 105 patients have received intraoperative radiotherapy at the National Cancer Institute. In a small randomized study on resectable pancreatic carcinoma, there appears to be an improvement in local control, disease-free and overall survival. In a small study of resectable gastric carcinoma, there additionally appears to be an improvement in local control, and a suggestion of improvement in survival. In patients with unresectable carcinoma of the pancreas, and large but resectable retroperitoneal sarcomas, there does not appear to be any improvement in local control or overall survival with the use of intraoperative radiation therapy combined with lower dose external beam radiation when compared to high dose external beam radiation. Pilot studies in patients with sarcomas of the bony pelvis show a suggestion in improvement in local control compared to historical controls. The pilot trial of intraoperative radiation therapy in locally advanced lung carcinoma has only entered 4 patients and is too early to assess any significant results.

Significance to Biomedical Research and the Program of the Institute

The intraoperative radiation therapy studies carried out in the Radiation Oncology and Surgery Branches are the first prospective randomized trials looking at the role of this innovative way of delivering radiation therapy as a means of improving local control while reducing local toxicity. We have been a leader in developing various technical modifications to the delivery of intraoperative radiation therapy.

Proposed Course

We plan to continue the pilot trials and the randomized trial in gastric carcinoma. Within the near future, we will embark on a randomized prospective trial in locally advanced rectal carcinoma.

Publications

1. Sindelar, W.F., Hoekstra, H., Restrepo, C., Kinsella, T.J.: Pathological Tissue Changes Following Intraoperative Radiotherapy. Am. J. Clin. Oncol. 9: 504-509, 1986.

2. Tepper, J.E., Gunderson, L.L., Goldson, A.L., Kinsella, T.J., Shipley, W.H., Sindelar, W.F., Wood, W.C., Martin, J.K.: Quality Control Parameters of Intraoperative Radiation Therapy. Int. J. Radiat. Oncol. Biol. Phys. 12: 1687-1695, 1986.

3. Barnes, M., Pass, H., DeLuca, A., Tochner, Z., Potter, D., Terrill, R., Sindelar, W.F., Kinsella, T.J.: Response of the Mediastinal and Thoracic Viscera of the Dog to Intraoperative Radiation Therapy (IORT). Int. J. Radiat. Oncol. Biol. Phys. 13: 371-378, 1987.

4. Pass, H.I., Sindelar, W.F., Kinsella, T.J., DeLuca, A.M., Barnes, M., Kurtzman, S., Hoekstra, H., Tochner, Z., Roth, J., Glatstein, E.: Delivery of Intraoperative Radiation Therapy (IORT) After Pneumonectomy: Experimental Observations and Early Clinical Results. Ann. Thor. Surg. (in press), 1987.

5. Hoekstra, H.J., Restrepo, C., Kinsella, T.J., Sindelar, W.F.: Histopathological Effects of Intraoperative Radiotherapy on Pancreas and Adjacent Tissues: A Postmortem Analysis. J. Surg. Oncol. (in press), 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06358-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Effects of Gamma-Irradiation on Cells and Their Constituents

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

P.I.:	P. Riesz	Research Chemist	ROB,NCI
Others:	Murali Krishna Cherukuri	Visiting Fellow	ROB,NCI
	T. Kondo	International Research Fellow	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	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.)*

Certain water soluble sulfonated phthalocyanines are efficient photosensitizers for killing mammalian cells and appear to be promising candidates for replacing hematoporphyrin derivative in photodynamic therapy (PDT) of human tumors. The post-illumination photohemolysis of human red blood cells was used as a model system for studying membrane damage sensitized by various sulfonated phthalocyanines. Scavenger studies with tryptophan (which reacts rapidly with singlet oxygen) and mannitol (which reacts rapidly with hydroxyl radicals but not with singlet oxygen) are consistent with the predominant role of singlet oxygen in the photohemolysis sensitized by sulfonated aluminum phthalocyanine. Similar results have been obtained in preliminary experiments for the photosensitized cell killing of V-79 Chinese hamster cells. The chemical effects of ultrasound are being investigated in relation to hyperthermia (used in combination with radiation therapy) and the possible effects of diagnostic and therapeutic applications. In the process of transient cavitation, the collapsing cavitation bubbles result in very high temperatures (> 5000° C) and pressures which lead to the dissociation of water to hydrogen atoms and hydroxyl radicals. For solutions containing volatile compounds such as alcohols, the reaction products are those typical of combustion processes (in the collapsing bubbles) and those similar to the effects of ionizing radiation due to the hydrogen atoms and hydroxyl radicals which escape into the bulk of the solution to react with solute molecules. In the case of methanol, methyl radicals formed by the high temperature dissociation of the carbon-oxygen bond, as well as radicals formed by the reactions of hydrogen atoms and hydroxyl radicals with methanol were detected by spin trapping and electron spin resonance. The radicals produced in the sonochemistry of nucleic acid bases, nucleosides, amino acids and peptides have been identified by spin trapping.

GPO 914-916

Project Description

Objectives: The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals, or by the "indirect effect" where radical species are hydrated electrons, hydrogen atoms and hydroxyl radicals.

In the chain of events that lead to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells; (b) halogenated pyrimidines which are incorporated into DNA; and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells. Studies of the mechanism of action of radiosensitizers and radioprotectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

In the last few years, it has become apparent that superoxide anion radicals and hydroxyl radicals are found in many biological systems in the absence of either ionizing radiation or UV-photolysis. Recent reports have indicated that radicals are produced in the presence of certain anti-cancer drugs such as Bleomycin and Adriamycin. The significance of radical reactions is therefore not confined to radiation biology. It has also been shown that damage to tissues following ischemia appears to occur during reperfusion with oxygenated blood. This damage is generally considered to be due to the excessive production of superoxide radicals and hydrogen peroxide. In support of this hypothesis, it has been shown that in several model systems superoxide dismutase, catalase or allopurinol (a xanthine oxidase inhibitor) protect ischemic tissue from oxidative damage during reperfusion.

Methods Employed

Nucleic acids, proteins and their constituents were gamma-irradiated either in the solid state or in aqueous solutions in a 800-curie Colbalt gamma-source. Electron spin resonance studies were carried out with a Varian E-9 Spectrometer connected to an IBM-XT computer. For photolysis studies at specific wavelengths, a 1000-watt high pressure Xenon arc source and monochomator were employed. For ultrasound exposures, aqueous solutions were insonated in a non-perturbing cylindrical cell with 1 mil mylar windows in an anachoic ultrasound exposure apparatus at 30 $\pm$ 0.5 degrees C. Specimens were exposed to either continuous wave or tone bursts if 1 MHz ultrasound to simulate both therapeutic and diagnostic exposure conditions. In the spin trapping method, the short-lived free radicals react with a diamagnetic scavenger (the spin trap) to produce longer-lived radicals (the spin adduct) which can be conveniently investigated by e.s.r. In our studies, 2-Methyl-2-Nitrosopropane and 5,5-Dimethyl-1-Pyroline-1-Oxide were employed as the spin traps.

Major Findings

I. The Role of Singlet Oxygen in the Photohemolysis of Red Blood Cells Sensitize by Phthalocyanine Sulfonates (with M. Sonoda and Murali Krishna Cherukuri)
The post-illumination photohemolysis of human red blood cells was used as a model system for studying membrane damage sensitized by various phthalocyanine sulfonates. The observed deuterium isotope effect with the aluminum compound and the large effects of tryptophan are consistent with a predominant role of singlet oxygen in photohemolysis.

II. Photosensitization of V-79 Chinese Hamster Cells by Aluminum Phthalocyanine Sulfonate (with J.B. Mitchell and A. Russo)
Preliminary scavenging experiments with L-tryptophan and mannitol indicate the predominant role of singlet oxygen in cell killing. The similar effects of L- and D-tryptophan are consistent with singlet oxygen scavenging and show that the effect of tryptophan does not involve its participation in metabolic pathways of the cells.

III. Free Radical Generation by Ultrasound in Aqueous Solutions of Nucleic Acid Bases and Nucleosides (with T. Kondo and Murali Krishna Cherukuri)
Direct evidence for the sonochemical formation of intermediate radicals of nucleic acid components (pyrimidine bases and nucleosides) in argon-saturated aqueous solutions was obtained. ESR and spin trapping indicated that the major spin trapped radical of thymine and thymidine was the 5-yl radical and that of cytosine, cytidine, uracil and uridine was the 6-yl radical.

IV. Sonochemistry of Amino Acids and Peptides (with Murali Krishna Cherukuri and T. Kondo)
The intermediate radicals in the sonochemistry of various amino acids and peptides were identified by spin trapping and ESR and compared with those formed by hydroxyl radicals generated by photolysis of hydrogen peroxide containing solutions of amino acids and peptides.

V. Sonochemistry of Aqueous Alcohol Solutions (with Y. Lion, Murali Krishna Cherukuri and T. Kondo)
The room-temperature sonochemistry of aqueous solutions containing volatile constituents such as alcohols leads to the formation of products similar to those of combustion chemistry formed at the very temperature (> 5000°C) and pressures in collapsing cavitation bubbles, plus products similar to those generated in aqueous radiation chemistry formed by the escape of hydrogen atoms and hydroxyl radicals from the collapsing cavities into the bulk of the solution. By ESR and spin trapping, it was possible to identify the combustion products from methanol (methyl radicals formed by scission of the very strong C-O bond), as well as radicals formed by the reaction of H and OH with methanol. Methanol, ethanol, isopropanol and n-propanol were investigated, and new unusual deuterium exchange reactions were discovered.

VI. A New ESR Method for Detecting Oxygen Radicals in Biological Membranes (with I. Rosenthal, FDA, Chemical Contaminant division, Center for Food Safety and Nutrition; Murali Krishna Cherukuri and T. Kondo)

Preliminary studies indicate that certain sterically hindered cyclic secondary amines can be converted by hydroxyl radicals in the presence of oxygen to stable, ESR-observable introxide radicals. The chemistry for the incorporation of such amines into long chain fatty acids at different positions along the chain is known. Consequently, it appears likely that such compounds could be used for the ESR detection of oxygen radicals formed in lipid peroxidation of mammalian cells.

Significance to Biomedical Research and the Program of the Institute

Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy; (2) carcinogenesis; (3) stability of the genetic pool; (4) the suppression of the immune mechanism; and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal cells, and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained.

Proposed Course

To continue studies on the effects of ionizing radiation on mammalian cells and macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be investigated. New areas of interest include photosensitized cell killing by porphyrins and phthalocyanines in relation to photodynamic therapy and chemical and biological effects of ultrasound.

Publications

1. Riesz, P., and Christman, C.L. Sonochemical free radical formation in aqueous solutions. Fed. Proc. 45: 2485-2492, 1986.

2. Decarroz, C., Wagner, J.R., Van Lier, J.E., Murali Krishna, C., Riesz, P., and Cadet, J. Sensitized photooxidation of thymidine by 2-methyl-1,4-naphtoquinone. Int. J. Radiat. Biol. 50: 491-505, 1986.

3. Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B., and Riesz, P. The distinction between exo- and endocellular spin trapping of oxygen radicals. In: Rotilio, G.V. (Ed.): "Superoxide and Superoxide Dismutase." Rome, 1986, pp. 119-121.

4. Rosenthal, I., Murali Krishna, C., Riesz, P., and Ben-Hur, E. The role of molecular oxygen in the photodynamic effect of phthalocyanines. Radiat. Res. 107: 136-142, 1986.

5. Samuni, A., Carmichael, A., Russo, A., Mitchell, J.B., and Riesz, P. On the spin trapping and ESR detection of oxygen derived radicals generated inside cells. Proc. Natl. Acad. Sci. USA 83: 7593-7597, 1986.

6. Murali Krishna, C., Lion, Y., and Riesz, P. A study of $^1O_2$ production by immobilized sensitizer outside the solution. Measurement of $^1O_2$ generation. Photochem. Photobiol. 45: 1-6, 1987.

7. Christman, C.L., Carmichael, A.J., Mossoba, M.M. and Riesz, P. Evidence for free radicals produced in aqueous solutions by diagnostic ultrasound. Ultrasonics. 25: 31-34, 1987.

8. Murali Krishna, C., Decarroz, C., Wagner, J.R., Cadet, J., and Riesz, P. Menadione sensitized photooxidation of nucleic acid and protein constituents. An ESR and spin-trapping study. Photochem. Photobiol. (in press).

9. Rosenthal, I., and Riesz, P. Electron spin resonance and spin trapping studies of radiation damage in biologically significant molecules. Radiat. Phys. Chem. 1987 (in press).

10. Sonoda, M., Murali Krishna, C., and Riesz, P. The role of $^1O_2$ in the photohemolysis of red blood cells sensitized by phthalocyanine sulfonates. Photochem. Photobiol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06359-04 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phase I/II Study Iododeoxyuridine (NSC39661) Given as an Intravenous Infusion

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

PI:	T. J. Kinsella	Deputy Branch Chief	ROB, NCI
Others:	E. Glatstein	Chief	ROB, NCI
	J. Rowland	Nurse	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Therapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

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

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

The Radiation Oncology Branch has continued studies using iododeoxyuridine given by continous intravenous infusion for 24 hours daily for up to 14 days in patients with high-grade primary brain tumors and other poorly radioresponsive tumors. The treatment protocol involves two separate, two-week infusions of IUdR combined with twice daily radiation therapy. Approximately 60 patients have been entered on the trial using the continuous intravenous infusion, including 36 patients with glioblastoma multiforme. The maximal tolerable dose of IUdR given as a continuous intravenous infusion for 24 hours a day for up to 2 weeks, is 1000 mg/m^2/day. Dose-limiting systemic toxicity is thrombocytopenia. There appears to be an improvement in median survival in glioblastoma patients compared to historical controls with a median survival approaching 14 months. Additionally, in patients with large, unresectable sarcomas, local control has been achieved in 12 of 15 patients. Again, this represents an improvement in local control of large, unresectable sarcomas compared to historical controls.

Project Description

Professional Personnel Engaged on the Project:

R. Klecker	Technician	CPB, NCI
P. Spaeth	Research Associate	CPB, NCI
J. Collins	Senior Investigator	CPB, NCI
D. Wright	Senior Investigator	SN, NINCDS

Objective: This is a Phase I/II study designed to assess the toxicity and treatment results using IUdR as a clinical radiosensitizer given as a continuous intravenous infusion for 24 hours/daily combined with high dose, hyperfractionated irradiation. Local (within the radiation field) and systemic toxicity are closely evaluated in patients with poorly radioresponsive tumors. Pharmacokinetics of the drug is being studied in collaboration with the Clinical Pharmacology Branch. Incorporation of IUdR has also been determined in circulating granulocytes using an HPLC technique developed in the Clincal Pharmacology Branch. Finally, incorporation of the drug into human tumors is being studied in selective patients by tumor biopsy using monoclonal antibody to IUdR.

Methods Employed

Patients with histologically confirmed Grade IV gliomas and other patients with locally advanced non-CNS tumors are eligible for this Phase I/II study. Eligibility criteria include normal peripheral blood counts, normal renal function, and a life expectancy of at least 2 months. Patients must also be ambulatory and able to be treated as out-patients. The IUdR is infused using a flexible silastic catheter placed in the superior vena cava. A portable, automatic infusion pump maintains a constant infusion over the 24-hour duration. Twice daily radiation therapy is begun within 7 to 10 days of the IUdR infusions.

Major Findings

We have determined the maximum tolerable dose of IUdR given as a 24-hour infusion for 14 days. The MPD is 1000/gm/m^2. The major systemic toxicity is thrombocytopenia. Moderate myelosuppression is also seen. Bone marrow recovery occurs within a period of 7 to 10 days after stopping the infusion and tolerance to the second infusion is similar to the first. Similar patients have developed mild to moderate stomatitis. No skin toxicity has been seen. Steady state plasma levels of IUdR are in the range of 3 to 4 x 10^6 molar/liter, using the infusions of 1000/mg/m^2. Preliminary analysis of patients treated with high grade glioma shows suggestion of an improvement in median survival in the range of 14 months compared to historical controls with radiation therapy alone in the range of 9 months.

Significance to Biomedical Research and the Program of the Institute

These studies on the use of halogenated pyrimidine analogs as clinical radiosensitizers open up new avenues for improving radiation response in traditionally poorly responsive tumors. Our work has stimulated a considerable amount of interest nationally, and other institutions are now engaged in clinical studies of these sensitizers based on our results.

Proposed Course

We have essentially completed the Phase I/II study of intravenous Iododeoxyuridine given as a 24-hour infusion. A new Phase I/II study of combining iododeoxyuridine and fluorodeoxyuridine has been initiated. FUdR can increase incorporation of IUdR into the DNA by decreasing intracellular thymidine via inhibition of thymidine synthetase. A second Phase I/II clinical study is being designed to combine intravenous IUdR with interstitial brain implants in patients presenting with small accessible high-grade gliomas. In vitro results from our laboratory showing ability to sensitize V79 cells with low dose rate irradiation similar to the clinical situation of interstitial implants.

Publications

1. Mitchell, J.B., Russo, A., Kinsella, T.J., and Glatstein, E.: The use of non-hypoxic cell sensitizers in radiobiology and radiotherapy. Int. J. Rad. Oncol. Biol. Phys. 12: 1513-1518, 1986.

2. Kinsella, T.J., Dobson, P., Mitchell, J.B.: Interaction of iododeoxyuridine (IdUrd) and its primary metabolite, iodouracil (IUra) on radiation response. Int. J. Rad. Oncol. Biol. Phys. 12: 1519-1522, 1986.

3. Belanger, K., Klecker, R., Rowland, J., Kinsella, T.J., Collins, J.M.: Incorporation of Iododeoxyuridine (IdUrd) into Cellular DNA in Patients Receiving Continuous Intravenous Infusions. Cancer Res. 46: 6509-6512, 1986.

4. Kinsella, T.J., Glatstein, E.: Clinical Experience with Intravenous Radiosensitizers in Unresectable Sarcomas. Cancer 59: 908-915, 1987.

5. Jackson, D., Kinsella, T., Rowland, J., Wright, D., Katz, D., Main, D., Collins, J., Kornblith, P., Glatstein, E.: Halogenated Pyrimidines as Radiosensitizers in the Treatment of Glioblastoma Multiforme. Amer. J. Clin. Oncol. (In press) 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06360-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Radionuclide Generators to Produce the Iridium-194 Beta Emitter

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

P.I.:	Saed Mirzadeh	Expert	ROB, NCI
Others:	Otto A. Gansow	Senior Investigator	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Inorganic and Radioimmune Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The project investigates the design, manufacturing, testing, and use of a novel radionuclide generator for biomedical applications. The generator has a long shelf-half-life, many years, and produces a 20 hour radionuclide daughter which emits high-energy beta particles that have suitable characteristics for labelling proteins through bifunctional chelates.

In the $^{194}Os/^{194}Ir$ generator containing the parent nuclei, ^{194}Os, has a half-life of 6.0 years which beta-decays, E^{max}_{β}=100 keV, to the 19.15-hour ^{194}Ir daughter. The ^{194}Ir daughter decays with the emission of 2.2 Mev beta particles to the ground state of ^{194}Pt (86%) and to the 328.5 keV first excited state with emission of 1.9 MeV beta particles (9.2%). There is a 328.5 keV first excited state with emission of 1.9 MeV beta particles (9.2%). There is a 328.5 keV gamma-ray which follows the decay of ^{194}Ir with 13% absolute aboundance. The absence of high intensity gamma-rays in the decay of ^{194}Ir, with the exception to the 328.5 keV, makes this beta emitter nuclei very attractive from the point of view of dosimetric considerations. On the other hand, the presence of 328.5 keV gamma-rays, makes ^{194}Ir a superior nucleus to ^{90}Y for tumor imaging.

Preliminary calculations indicate that several mCi of the parent, ^{194}Os, can be produced in a nuclear reactor by double neutron capture of an ^{192}Os (natural aboundance of 41%) target. By using enriched ^{192}Os, a two fold increase in the yield results and also reduces the production of impurities. The enriched Osmium-192 with enrichment factor of greater than 99% is purchased from Oak Ridge National Laboratory.

Project Description

Objective: To develop a radionuclide generator system which will produce ^{194}Ir for attachment to proteins through bifunctional chelations.

Methods Employed

Osmium-194 is produced in a nuclear reactor by irradiating enriched Os-192. After irradiation, Os target is dissolved, purified and loaded into a suitable chromatographic column. The daughter ^{194}Ir is eluted with suitable solvents. The yield of the elution of the daughter, the breakthrough of the parent, radiation and chemical resistance of the column generator, radiochemical and radionuclidic purity of the product are being investigated.

Significance to Biomedical Research and the Program of the Institute

The developement of the $^{194}Os/^{194}Ir$ generator for production of 20 hour ^{194}Ir would increase access of the biomedical community to a high-energy beta emitter as radiotherapeutic agent.

Proposed Course

1) Development of $^{194}Os/^{194}Ir$ generator.
2) Prepare for suitable bifunctional chelates for attachment of ^{194}Ir for proteins.
3) Study proteins labeled with ^{194}Ir in vitro and in vivo.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06361-03 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Phototherapy of Murine Ovarian Cancer by Hematoporphyrin Derivative

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

P.I.:	A. Russo	Clinical Associate	ROB,NCI
Others:	J. B. MItchell	Senior Investigator	ROB,NCI
	J. Fisher	Chemist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	3	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 use of hematoporphyrin derivative (HPD) in combination with read light is currently under investigation as an anti-cancer treatment modality. A major advantage of this therapy is the purported selectivity of tumor versus normal tissue response. Studies have been designed and are currently underway to establish HPD retention in tumor versus normal tissue in a murine model. An ovarian tumor model originating from a mouse has been used to determine the pharmacodynamic of HPD, optimization of HPD delivery, laser penetration, dose and timing of light and drug delivery into the peritoneal cavity. The use of different wavelengths of light is being studied on the ascites tumor model. Human xenografts from ovarian and lung are being studied in nude mice. Measurement of singlet oxygen is being studied to prepare the way for dosimetry. Dog toxicity to intracavitary HPD and laser light is being studied. The effects of phototherapy on hematologic components is being studied with intent of purifying blood of AIDS and hepatitis viruses as well as different ineffective parasites.

Project Description

Objective: Establish a laboratory model for treatment of ovarian and lung cancer by HPD and light. Establish in an animal model that singlet oxygen can be measured. to determine the toxicity of intracavitary light. To determine the best means of scattering light.

Methods Employed

An ovarian cancer murine model which grows in the peritoneal cavity is being used. Endpoints utilized will include tumor response, survival time and in vitro survival. Light will be delivered via fiber optics and quantitation of HPD will be determined spectroscopically. Athymic nude mice will be the recipients of human ovarian tumor (3) and human lung cancer (2). Dogs are subjected to either ip or ip/iv injection of HPD and then treated with ip laser light. Singlet oxygen is being measured at 1270nm using outer phase quatrature techniques and Fourier Transform time averaging. Screen photosensitizing agents that have the photochemical, physical, and biological characteristic to localize in tumors as well as finding agents that bind to nucleic acids. This will entail plasmid destruction followed by agarose gel chromatography.

Major Finding

Differential retention of HPD, murine ascites tumor can be cured by this technique, laser therapy to the ip surface does not appear to have toxicity in mice and preliminary singlet oxygen has been measured in an animal system for the first time. The canine model has shown that there is minimal, but expected, damage to the superficial aspect of the liver. Transaminase levels increased immediately after intrabdominal treatment and quickly return to normal.

Significance to Biomedical Research and the Program of the Institute.

The ROB will soon become involved clinically in phototherapy and these studies should provide better insight into the dynamics of HPD therapy.

Proposed Course

Continue to explore the model human xenografts, develop dosimetry techniques, explore the mechanisms of HPD/light cytotoxicity, modulate the tumor response, alter HPD metabolism, synthesize new sensitizer dyes, utilize porphyrin imaging techniques to determine when to treat with HPD/light.

Publications

1. Tochner, Z., Mitchell, J.B., Smith, P., Harrington, F., Glatstein, e., and Russo, A.: Photodynamic therapy of ascitic tumor within the peritoneal cavity. British Journal Cancer, 53: 733-736, 1986.

2. Tochner, Z., Mitchell, J.B., Smith, P., Harrington, F., Russo, d., and Russo, A. Phototherapy of ascites tumors: Canine Toxicity Study. Laser in Medicine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06363-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)*

P.I.:	A. J. Fornace, Jr.	Cancer Expert	ROB, NCI
Others:	T. J. Kinsella	Deputy Branch Chief	ROB, NCI

COOPERATING UNITS *(if any)*

Applied Genetics, Freeport, New York (D. Yarosh).

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	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.)*

R. S. Day, III, D. Yarosh, 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 Alkylguanine Alkyltransferase. We have been able to partially purify the enzyme from human liver. This enzyme removes alkylation damage at the 06 position of guanine but not at other sites in DNA. We plan to further characterize this enzyme and purify it to the point where amino acid sequence information is obtained. In collaboration with D. Yarosh, human liver extracts are being used to isolate adequate quantities of this protein. Differences in the transcription of specific RNAs in mer^+ and mer^- cell lines were investigated. With hybridization subtraction, a cDNA library was constructed from a mer^+ cell which had been depleted of sequences common to matched mer^- cell lines. However, when other mer^+ and mer^- lines were used, this correlation was not consistent - i.e., none of the cDNA clones coded for a transcript consistently more abundant in mer^+ compared to mer^- cell lines.

Project Description

Objective: To characterize the human alkylguanine alkyltransferase enzyme and ultimately isolate the gene.

Methods Employed

Standard molecular biology and protein biochemistry approaches.

Major Findings

A consistent cDNA clone absent in mer$^-$ cells was not detected. We are also approaching this subject by characterizing the enzyme.

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 carcinogenesis and cancer treatment.

Proposed Course

See summary.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06365-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

RNA Transcripts Induced by Hyperthermia in Rodent Cells

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

P.I.:	A. J. Fornace, Jr.	Cancer Expert	ROB, NCI
Others:	J. B. Mitchell	Radiobiologist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	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.)*

Prokaryotic and euklaryotic cells respond to environmental stress by the induction of a variety of stress-related proteins. In mammalian cells, the most well characterized group of stress proteins are induced by hyperthermia. Transcription of heat shock proteins increases markedly after hyperthermia and several of these genes have been cloned from HeLa cells in other laboratories. It is likely that transcription of other genes is also induced in mammalian cells since approximately 10-20 genes are induced in prokaryotes and lower eukaryotes. One approach to isolate such transcripts is to enrich for heat shock specific cDNA's by hybridization subtraction with mRNA from control cells. We have done this with rodent cells, V79, and we have also constructed a cDNA library from heat shock treated (HS) cells. Our results indicate that the most abundant transcript induced by HS in V79 and CHO cell lines is a small repetitive genetic element. Sequence analysis of cDNA lines revealed that they were short RNA polymerase III transcripts of the rodent B2 repetitive element. Transcription of this repetitive element has previously been found to be increased in transformed rodent cells and rodent tumor cells. It is of interest that HS proteins are usually elevated in transformed cells. The function of this B2 RNA is unknown, but it is polyadenylated with localization to the cytoplasm; it may play some role in the regulation of translation. This hypothesis is supported by our observation that a different spectrum of B2 transcripts are transcribed after heat shock. Heat shock has profound effects on translation; e.g., preferential translation of heat shock protein RNA.

We have also found that the induction of B2 RNA by heat shock correlated temporarily with the induction of the major heat shock protein RNA's.

Project Description

Objective: To identify genes induced by hyperthermia in V79 cells and to study their regulation.

Methods Employed

Standard molecular biology approaches; hybridization subtraction procedures as used by Timberlake, Sargent, Davis, and others.

Major Findings

The major transcript induced by HS in V79 and CHO cells is from a repetitive genetic element. This sequence may play a role in the regulation of the heat shock response. cDNA clones for most of the major heat shock proteins have been isolated.

Significance to Biomedical Research and the Program of the Institute

A more thorough understanding of the response of mammalian cells to hyperthermia would benefit both clinical hyperthermia research and also how mammalian cells respond to environmental stress.

Proposed Course

Further characterization of this repetitive element induced by HS is currently underway. The cellular response at the level of transcription to various heat shock protocols will be studied and cellular parameters, such as thermotolerance, will be correlated. The response in mutant cells with increased sensitivity to hyperthermia will also be studied. Transcription of these genes after different types of stress, such as oxidative stress, will be studied.

Publication

1. Fornace, A.J., Jr., and Mitchell, J.B. Induction of B2 RNA polymerase III transcription by heat shock; enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. Nucleic Acids Research 14: 5793-5811, 1986

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06367-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Mechanisms of Radioprotection

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

P.I.:	A. Russo	Clinical Associate	ROB,NCI
Others:	J. B. Mitchell	Senior Investigator	ROB,NCI
	W. DeGraff	Biologist	ROB,NCI
	N. Friedman	Biologist	ROB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	3	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.)*

Thiols have long been studied as radioprotective compounds, yet the mechanism of protection is still poorly understood. We have developed means by which the major cellular thiol, glutathione, can be either depleted or elevated and the access radiosensitivity. We have shown that glutathione is not a major protector from the effects of ionizing radiation in mitotically active cells. Work is underway to synthesize compounds varying in chemical structure that may provide insight into the mechanism and requirements of radioprotection agents. The importance of membrane in specialized tissue such as lymphocytes is being studied. The relationship of membrane oxidation and protection from such oxidative damage is being studied. The importance of separate thiol dependent detoxification enzymes versus general oxidative detoxification is being studied. Additionally, by evaluation of previously synthesized radioprotectors using structure/activity relation calculations, we plan to evaluate already synthesized and tested compounds as a means to guide the synthesis of new compounds that may afford differential protection to normal versus tumor tissue.

Project Description

Objective: To develop normal tissue radioprotective agents.

Methods Employed

V79 hamster cells and conventional cell cloning assays will be used. Biochemical assays for assessing the redox potential of cells have been developed. In vivo murine systems will be employed when in situ biochemical activation is felt to be a requirement for unmasking of radioprotecting agents. GC/Mass spectroscopic analysis of C-5 fragments will be studied in Lymphoid cells being kept in vitro.

Major Finding

Cellular glutathione does not play a major role in the radiation response, either aerated or hypoxic. With lymphocyte population, the membrane is responsible for interphase death. Interphase death susceptibility can be correlated with oxidative detoxifying enzyme concentration.

Significance to Biomedical Research and the Program of the Institute

To provide a rational approach to radioprotective agents and to better understand the mechanism of radiation damage.

Proposed Course

Synthesize and evaluate new compounds. To continue exploring the mechanism of interphase death. To apply information gathered from resistant cells and apply this to normal human cell. Ultimately to apply this treatment of cancer and protection of normal tissue.

Publications

1. Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B., and Riesz, P:. On the spin trapping and ESR detection of oxygen-derived radicals generated inside cells. Proc. Natl. Acad. Sci. 83: 7593-7597, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06369-04 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Radiation Characteristics of a 22 MeV Medical Microtron

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

PI:	R. Miller	Radiation Physicist	ROB, NCI
Others:	J. van de Geijn	Radiation Physicist	ROB, NCI
	B. Chin Arora	Clinical Physicist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.8	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The Physics Section is continuing studies of the radiation characteristics of the Scanditronix MM-22 medical Microtron. Current research is centered around our Intraoperative Radiotherapy Program (IORT) and concerns the dosimetry of special IORT applicators. Associated with this is a study of the effects of using asymmetric electron collimation on the dose distributions of these applicators. A new color camera television system for viewing the intraoperative portal is under development. This system will employ a permanent mirror of aluminized Mylar instead of the current retractable glass mirror. This should allow remote viewing of the radiation portal during treatment.

Project Description

Objectives: The optimization of the radiation and operational characteristics of the Microtron.

Methods Employed

The high quality and versatile radiation measurement systems available to the Branch are used by staff personnel in cooperation with experts from the manufacturer to determine the basic performance of the various critical functions of the machine and its monitoring equipment. Several of these functions have been found less than optimal for the special purposes envisioned by the ROB and in several cases, dramatic improvements have been obtained already.

Major Findings

Several performance characteristics were unsatisfactory for our purposes: electron depth dose distribution and tranverse beam profiles, monitor characteristics. Interaction between representatives of our staff and experts from the firm have resulted already in performance characteristics much better than required by the specifications.

Significance to Biomedical Research and the Program of the Institute

The Microtron is to be utilized primarily for intraoperative radiotherapy. As such, its reliability and (especially) its beam characteristics are of critical importance to this program. Also, the machine offers some technical features such as independently adjustable collimator jaws, which are meaningful only with more than minimum performance characteristics.

Proposed Course

To be continued.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06370-03 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Optimization of Treatment Planning for Brain Implants

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Other:	R. Miller	Radiation Physicist	ROB, NCI
	E. Lamoreaux	Computer Specialist	ROB, NCI
	K. Yeakel-Orr	Dosimetrist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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 purpose of this work is to optimize physics, computer based and technical support for a brain implant protocol. In particular it aims at optimization of the chain of procedures comprising patient data acquisition, treatment planning, including optimal delineation of the target, determination of the number of radioactive sources, their strength and position, and determination of the surgical mechanical positioning devices and the further development and adaptation of locally developed computer programs, with emphasis on versatile imaging as a basis for optimization of irregular implants.

Project Description

Personnel:

T. Kinsella	Senior Investigator	ROB, NCI
D. Wright	Neuro-Surgeon	SN, NINCDS

Objective: To develop a computer-assisted system for optimizatin of the physical and technical aspects of radioactive seed implants in brain tumors; criteria are:

1. Accurate fitting of a critical dose rate surface around the chosen target volume, which may be a regular or irregular shape;

2. A uniform dose distribution inside the target; and

3. A minimum number of catheters positioned in the brain in the most economical and accurate way.

Methods Employed

1. Mathematical/Physical methods are employed to develop a generalized approach to seed placement and relative seed strength. The effort now concentrates on irregularly shaped tumors.

2. Computer-based image manipulation of diagnostic CT data to determine optimal access routes.

3. Development of mechanical positioning and directioning devices for use in data acquisition and in surgery.

Major Findings

Major results have been reached in the mathematical optimization, which is, partly for practical clinical reasons, in part computerized, in part manual-based,

so that a semi-automatic interactive computer based system will shortly be available. Considerable work needs to be done to make the system universal. An atlas is available for regular-shaped implants.

Significance to Biomedical Research and the Program of the Institute

The system is essential to technically and physically safe and radiation-economic patient treatment. The development aims at general applicability. The theoretical and computer related procedures apply to implants in general.

Proposed Course

Continuation.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06372-03 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Extension of the Net Fractional Depth Dose for Inhomogeneity Correction

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	B. Chin Arora	Radiation Physicist	ROB, NCI
	J. Pochobradsky	Computer Specialist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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 correction for the effect of inhomogeneity in body tissue is of considerable importance in the treatment optimization especially, of lesions in the thoracal region, such as the tumors of the breast, the esophagus, or the lung. The advent of CT has made this meaningful. The recently described NFD has been extended by the incorporation of the density scaling concept in the field size as well as the depth parameter, in the analytical formalism, thus including correction for inhomogeneities. This method now includes correction for the effect of secondary electron transport for a significant set of conditions. Further work needs to be done to incorporate the electron transport correction near the beam edges and for small inhomogeneities in larger fields. The method appears to be accurate, versatile and easy to implement.

Project Description

Objective: Accurate analytical description of the dose distribution in inhomogeneous media for the photon energies ranging from 4-20 MV, under clinically relevant conditions, i.e., including effects of secondary electron transport near beam edges and for small inhomogeneities.

Methods Employed

The NFD formalism has been extended by the introduction of the density scaling theorem: in principle, replacing the field parameter (the side of the equivalent square) by pc, p being the relative electron density, and the depth by the radiologic depth. A simple solution has been introduced to deal with secondary electron transport.

Major Findings

No new parameters or new coefficients are required. Agreement with measured data are excellent, and often superior to existing methods. The method has been implemented into the beam treatment program and is of considerable importance when using high energy x-ray beams in the thorax.

Significance to Biomedical Research and the Program of the Institute

1. The new method is more accurate than other existing "global" methods.

2. It improves the accuracy of computed dose distributions generated for tumors in the thoracal region, especially for the higher x-ray energies, when using small or narrow beams.

3. It appears that the "effective depth" method overestimated the dose to lung for breast treatment, but is reasonably accurate in the unit density areas.

4. The method is easily implemented.

Proposed Course

Continued verification for various beam energies in clinically significant conditions. Extension to include correction near beam edges and edges of small inhomogeneities.

Publications

1. McKenna, W.G., Yeakel, K., Klink, A., Fraass, B.A., van de Geijn, J., Glatstein, E., Lichter, A.S. Is Correction for Lung Density in Radiotherapy Treatment Planning Necessary? Int. J. Radiat. Oncol. Biol. Phys. 13:273-278, 1987.

2. van de Geijn, J.: The extended net fractional depth dose: Correction for inhomogeneities, including effects of electron transport in photon beam dose calculation. Medical Physics 14:84-92, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06374-03 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Effect of Radioprotectors and Radiosensitizers on DNA Damage Produced by X-rays

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

P.I.:	A. J. Fornace, Jr.	Cancer Expert	ROB, NCI
Others:	T. J. Kinsella	Deputy Branch Chief	ROB, NCI
	J. B. Mitchell	Radiobiologist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Cellular radiosensitivity is affected by many factors which may be clinically important. For example, cellular oxygen concentration and the redox potential of the cell affect cellular radiosensitivity and probably are also important clinically. Several agents, BSO or SR 2508, have been shown to decrease the effective sulfhydryl concentration in the cell and to act as hypoxic cell sensitizers. Addition of cysteamine, on the other hand, protects cells x-irradiated under oxic conditions. With alkaline and neutral elution, we have measured the effect of these agents on x-ray induced DNA damage, in particular, DNA double strand breaks (DSB), single strand breaks (SSB), and base damage (ESS). We have found that hypoxic irradiation markedly reduces the efficiency of DSB and SSB production by x-rays in V79 cells and reduces the yield of ESS to a lesser extent. When both BSO and SR 2508 were present during hypoxic irradiation, it markedly increased the yield of SSB and DSB, and had a lesser effect on ESS. The radioprotector cysteamine produced a marked decrease in the yield of DBS with x-rays, had a lesser effect with SSB, and little or no effect of ESS. The yield of DSB was most affected by hypoxic irradiation, the addition of radiosensitizers, or the radioprotector cysteamine. Although the lethal lesion produced by x-rays is unknown, indirect results of other indicate that DSB may be the critical lesion. Our work with radiosensitizers and radioprotectors support this hypothesis.

Project Description

Objective: To study the effect of radioprotectors and radiosensitizers on particular types of DNA damage.

Methods Employed

Alkaline and neutral elution. Standard cell culture techniques.

Major Findings

The efficiency of production of particular types of DNA damage, especially double strand breaks, by x-irradiation of mammalian cells is affected by hypoxic irradiation, the hypoxic cell radiosensitizers SR 2508 and BSO, and the radioprotector cysteamine.

Significance to Biomedical Research and the Program of the Institute

This work shows that agents which affect cellular radiosensitivity affect the production of different types of DNA damage. Such studies may aid in the development of effective radiosensitizers.

Proposed Course

Studies will be continued on the effect of various radiosensitizers and combination of radiosensitizers on x-ray induced DNA damage. The effect of such agents on the x-ray DNA damage in vivo will be considered.

Publications

1. Kinsella, T., Dobson, P.B., Russo, A., Mitchell, J.B., and Fornace, A.J., Jr. Modulation of x-ray DNA damage by SR-2508 + buthionine sulfoximine. Int. J. Radiat. Oncol. Biol. Phys. 12: 1127-1130, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06375-03 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

DNA Damage in X-irradiated Cells Treated with Halogenated Pyrimidines

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

PI:	T. J. Kinsella	Deputy Branch Chief	ROB, NCI
Others:	A. J. Fornace, Jr.	Cancer Expert	ROB, NCI
	J. B. Mitchell	Radiobiologist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.15	.05	.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.)*

Treatment of patients with halogenated pyrimidines, in particular IUdR, has been found to increase tumor radiosensitivity. In mammalian cells, BUdR or IUdR pre-treatment increases their radiosensitivity in vitro. We have found that the initial level of DNA single strand and double strand breaks induced by x-rays in V79 rodent cells is increased in cells pre-treated with IUdR or BUdR. At clinically relevant doses, we have found that both DNA single strand breaks and DNA double strand breaks are increased approximately two-fold when 25% of the thymine bases are replaced with IUdR or BUdR. This level of substitution was obtained with a 10 uM dose of IUdR - a concentration which can be achieved in vivo.

GPO 914-918

Project Description

Objective: To determine the effect of DNA substitution with halogenated pyrimidines on DNA damage in x-irradiated cells.

Methods Employed

Alkaline and neutral elution. Standard cell culture techniques.

Major Findings

DNA single strand and double strand breaks were substantially increased in cells pre-treated with concentrations of halogenated pyrimidines which can be achieved in the blood clinically.

Significance to Biomedical Research and the Program of the Institute

This work demonstrates that increased levels of DNA damage can be detected in cells treated with clinically relevant doses of x-ray and with clinically achievable concentrations of halogenated pyrimidines. This work should provide a basis for in vivo studies on the effects of halogenated pyrimidines on radiosensitivity.

Proposed Course

We plan to further characterize the effect of varying concentrations of halogenated pyrimidines on cellular DNA damage produced by x-rays. This approach can be adapted for in vivo studies, and eventually we should be able to monitor DNA damage and repair in clinical specimens and determine the effect of IUdR.

Publications

1. Kinsella, T.J., Dobson, P.P., Mitchell, J.B., Fornace, A.J.: Enhancement of X-ray Induced DNA Damage by Pretreatment with Halogenated Pyrimidine Analogs. Int. J. Radiat. Oncol. Biol. Phys. 13: 733-739, 1987.

2. Miller, R.W., DeGraff, W., Kinsella, T.J., Mitchell, J.B.: Evaluation of Incorporated Iododeoxyuridine Cellular Radiosensitization by Photon Activation Therapy. Int. J. Radiat. Oncol. Biol. Phys. (in press), 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06377-02 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Optimization of Dose Distributions from Intraoperative Applicators

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	K. Yeakel-Orr	Dosimetrist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI
	T. Kinsella	Senior Investigator	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.2	.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.)*

Due to the high single fraction doses employed in intraoperative radiotherapy, it is important to minimize areas of high dose. These areas arise due to the the scattering relationship between the photon collimators and the applicator system. They may be non-symmetric due to either an asymmetric applicator design or to manufacturing tolerances of the applicator system resulting in the center of the applicator to be slightly different than the central ray of the accelerator. These dose distributions can be optimized to predetermined criteria by the use of an optimum field size for each combination of applicator and electron energy. The use of asymmetric collimators can further correct for nonuniformity in the dose distribution due to asymmetric design, applicator bevel angle, of applicator mis-alignment.

GPO 914-918

Project Description

Objectives: To optimize the dose distribution from applicators used to deliver intraoperative radiation therapy with high energy electron beams.

Methods Employes

1. To develop criteria for defining the optimum dose distribution.

2. To study the dose distributions from applicators as a function of collimator setting and energy in order to determine optimum field sizes for each energy.

3. To use asymmetric collimator settings to correct for non-uniformities in the radiation field due to applicator shape, non-concentric applicator position and applicator bevel angle.

Major Findings

The criteria for defining the optimum dose distribution based on the prescription isodose line have been developed. These have been used to determine optimum field sizes for the applicators currently used to deliver IORT at the NCI. The areas of high dose have been reduced by as much as 25% by this method. The use of decoupled collimators to produce asymmetric fields can restore symmetry to the dose distribution when it is affected by applicator shape or non-concentric position.

Significance to Biomedical Research

Most institutions performing intraoperative radiotherapy use a fixed collimator setting for each applicator regardless of energy. Some places use the same field size for all applicators as well. This clearly results in areas of high dose in the treatment which can result in doses 30%-40% greater than the prescription dose. Proper choice of field size can substantially reduce these "hot spots" resulting in a more uniform dose distribution. The techniques should reduce the incidence of patient complications due to exceeding normal tissue tolerance doses.

Proposed Course

Continue the investigations concentrating on bevelled applicators. It is possible that the use of asymmetric collimators can correct for some of the adverse effects that begin to occur as the bevel angle becomes steeper.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06378-02 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

QA of Treatment Delivery by Means of Overlayed Digitized Simulator and Portfilms

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	R. Miller	Radiation Physicist	ROB, NCI
	B. Chin Arora	Radiation Physicist	ROB, NCI
	J. Pochobradsky	Computer Specialist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.1	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 quality assurance of the consistency of radiation treatment delivery with the prescription is a continual concern, locally as well as nationally. The ROB already employs graticules projecting onto simulator films and corresponding portfilms. Now a project is being started to overlay differently processed digitized films to increase the quality of information as well as to decrease the volume of documentation to be retained. The system should be of great interest to inter-institutional studies as well.

Project Description

Objective: 1) To improve the quality of documentation on the proper implementation of beam treatment set-ups.

2) To condense the amount of documentation to be kept, and to increase its objectivity and exchangeability.

Methods Employed

1. Take x-ray films at the simulator, in the planned beam positions including graticules projected onto the films.

2. Similar procedure at the treatment machine, producing port films with graticules.

3. Digitize both categories of films taking care to use the same orientation, centering and magnification, with help of the graticules projected onto all films.

4. Apply appropriate computer enhancement of both simulator films and the corresponding port films.

5. Overlay technique, bring-out salient anatomical features, graticules, block delineation, etc.

6. Using computer do measurements of significant deviations.

7. Store the results and properly label.

Major Findings

Exploratory experiments have been encouraging

Significance to Biomedical Research and the Program of the Institute

1. Q.A. and verification will become much more efficient, self-contained and attractive to use.

2. Documentation will be much more compact and easier to use.

3. Q.A. of joint studies will be much easier and more objective.

Proposed Course

To be started soon.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06379-01 RO

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Phase I Study of Photodynamic Therapy For Surface Malignancies

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

PI:	T. F. DeLaney	Senior Investigator	ROB, NCI
Others:	E. Glatstein	Branch Chief	ROB, NCI
	A. Russo	Senior Investigator	ROB, NCI
	N. Fox	Nursing Clinician	ROB, NCI
	G. Thomas	Microbiologist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Therapy 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.)*

The Radiation Oncology Branch has entered fifteen patients onto this study of photodynamic therapy. The treatment protocol involves the intravenous administration of the Photophrin II™ preparation of the hematoporphyrin derivative, followed by delivery of light to the affected area. Light is directed at the tumor using optical fibers coupled to an argon pumped dye laser. When possible, laser Doppler blood flow measurements have been made on patients before and after treatment. Fifteen patients have been entered onto the protocol to date. Toxicity associated with treatment includes cutaneous photosensitivity, with one patient developing a first degree sunburn on his arms upon exposure to bright sunlight 78 days after drug infusion. Two patients with extensive recurrent breast cancer on the chest wall developed transient discomfort in the treatment area requiring narcotics for pain control. In 10 patients with recurrent disease involving the skin, complete responses have been obtained in one patient with head and neck cancer, one patient with breast cancer, and one patient with cutaneous lymphoma. Re-aeration of the lung has been achieved in two patients with lung collapse secondary to obstructing tumor in the main stem bronchus following clean up bronchoscopy to move necrotic tumor; a third patient is awaiting treatment at the time of this report. Light delivery into the peritoneal cavity at the time of exploratory laparotomy has been achieved in one patient using a specially designed light diffusing rod and a dilute solution of intralipid instilled into the peritoneal cavity at the time of surgery. Measurements of light distribution using sterile photodiodes in the abdominal cavity have been made. Preliminary analysis of the data would indicate that there is significant attenuation of light by hemoglobin present, an issue which will need to be addressed if adequate light delivery to the peritoneal surface is going to be achieved in this manner.

Project Description

Professional Personnel Engaged on the Project:

H. Pass	Senior Investigator	SB, NCI
W. Sindelar	Senior Investigator	SB, NCI
P. Smith	Laser Physicist	BEIB, DES
R. Bonner	Biophysicist	BEIB, DES
W. Travis	Senior Investigator	LP, NCI
A. Dwyer	Senior Investigator	RB, NCI

Objective

This is a Phase I study designed to assess the toxicity and efficacy of photodynamic therapy with Photofrin II™ in treatment of surface malignancies in patients with neoplasms that have failed previous treatment or for whom no phase II or III agents or studies are available. The responsiveness of different malignant tumors to treatment with photodynamic therapy is also being studied. The physical parameters of light distribution in tissue are being measured and an attempt is made to correlate this with treatment effects from photodynamic therapy as assessed by biopsies of treated areas. Changes in blood flow induced by photodynamic therapy during and after treatment are being studied both in patients and laboratory animals using laser Doppler blood flow velocimetry. Finally, efforts are under way to develop the technical expertise to measure the production of singlet-oxygen, which is thought to be the active cytotoxic moiety produced by the interaction of light activated photosensitizer and molecular oxygen, in the hopes of correlating this with responsiveness of tumors to photodynamic therapy.

Methods Employed

Patients with surface malignancies that have or have not had previous treatment with other modalities are eligible for this protocol. Included are patients with any malignancy on (or just underneath the surface of the skin, primary or metastatic), surface tumors of the gastrointestinal tract from the mouth to the anus, surface tumors within the trachea and bronchial tree, surface genitouinary urinary tumors of females, and malignant pleural or ascitic tumors stemming from the surfaces of the chest wall or peritoneum. Because Photofrin II™ is a porphyin, patients with porphyria of any type are excluded. As the drug is metabolized in the liver, patients with severe liver disease are excluded. Patients with a curative option for their stage of disease are not eligible. Patients treated to date include six patients with recurrent breast cancer, three patients with endobronchial obstruction from metastatic melanoma, colon and ovarian carcinomas, two patients with cutaneous recurrences of head and neck cancer, and one patient each with recurrent cutaneous lymphoma and melanoma. One additional patient with recurrent rectal cancer was entered on the protocol at the time of exploratory laparotomy for measurement of light distribution within the peritoneal cavity without any administration of the photosensitizer for therapeutic intent.

Major Findings

We have determined that photodynamic therapy has activity against a variety of malignant tumors, often in spite of extensive previous treatment. In this study, complete responses have been seen in patients with recurrent cutaneous breast cancer, head and neck cancer, and lymphoma. Treatment effects are confined to the area exposed to light. Conversely, treatment efficacy is limited by light penetration. For example, in the two patients with endobronchial obstruction, treatment effected re-opening of the bronchial lumen and re-aeration of the lung but both patients had persistent tumor beyond the range of light in the adjacent lung and mediastinum which required additional treatment with radiation to ensure long term disease control. The complete responses obtained to date have been durable, up to eight months in one patient with recurrent head and neck cancer. Recurrent breast cancers of an inflamatory nature with tumor extensively permeating dermal lymphatics appear difficult to control with this modality; the three patients treated with this problem have not had their disease controlled by treatment. Treatment of large areas with ulcerated tumors may be painful and require narcotics for symptomatic relief. Deeply pigmented lesions such as melanoma limit light delivery. Laser Doppler blood flow measurements appear to indicate that photodynamic therapy induces a transient marked increase in blood flow with the peak flow occuring about 10 minutes after the start of treatment. Blood flow in areas of treated tumor will then fall to below basal levels by 24 hours after therapy. The relationship of tumor blood flow to tumor necrosis is as yet undetermined. Blood flow measurements in experimental murine tumors receiving photodynamic therapy are currently in progress.

Signifance to Biomedical Research and the Program of the Institute

Photodynamic therapy represents a potentially useful mode of curative therapy for selected groups of patients with malignant disease. In particular, patients with a tumor that is accessible to light either by superficial, endoscopic or interstitial illumination may benefit from such treatment. We envision a possible use for this therapy in patients with carcinoma in situ of the urinary bladder, cancers involving the peritoneal or pleural surfaces, and selected skin cancers.

Proposed Course

We propose to begin treating superficial carcinomas of the urinary bladder with photodynamic therapy in the near future. We are interested in making additional measurements to assess whether a clinically useful light distribution can be achieved within the peritoneal cavity at the time of a laparotomy as a prelude to photodynamic therapy for peritoneal carcinomatosis. We would also like to test the efficacy of photodynamic therapy in patients with refractory pleural effusions. Long range plans also include examination of other photosensitizers in the laboratory which are activated by light with deeper tissue penetration and which may have less cutaneous photosensitivity.

Publications

1. DeLaney, T.F. and Glatstein, E.: Photodynamic Therapy of Cancer. Comprehensive Therapy (in press), 1987.

2. DeLaney, T.F.: Photodynamic Therapy. Magrath, I. (ed.): New Directions in Cancer Treatment (in press), 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06380-01 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)*

P.I.:	A. J. Fornace, Jr.	Cancer Expert	ROB, NCI
Others:	J. B. Mitchell	Radiobiologist	ROB, NCI
	T. J. Kinsella	Deputy Branch Chief	ROB, NCI

COOPERATING UNITS *(if any)*

Dr. Ian Hickson, University of Newcastle on the Tyne, U.K.

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	0.8	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.)*

Several inducible DNA repair genes have been well characterized in prokaryotic systems; in eukaryotes including mammalian cells, there is increasing evidence that similar events may be occur. Recently, we have shown that hybridization subtraction can be used to enrich for sequences induced only several fold by a particular cell treatment such as heat shock. Chinese hamster cells were UV-irradiated and cDNA was synthesized from the polyadenylated (polyA) RNA of these cells. This "UV" cDNA was hybridized with polyA RNA from unirradiated cells and the nonhybridizing cDNA was isolated. With this approach, UV-induced sequences were enriched for over 30 fold. With this enriched cDNA, a cDNA library was constructed. 50 different cDNA clones were isolated which were found to code for transcripts induced 2 to 30 fold after UV. 27 different cDNA clones were sequenced and only metallothionein was identified in the GenBank database which indicates that most of these sequences have not been isolated before. In preliminary experiments, several of these clones were found to hybridize to UV-inducible transcripts in human fibroblasts. In Chinese hamster somatic cell mutants selected for increased sensitivity to DNA damaging agents by I. Hickson, several examples of both increased and decreased transcription in the mutant cells were found. The function of these cloned sequences is unknown, but it is probable that the protein products of at least some of these transcripts play a role in the cellular response to UV damage.

Project Description

Objective: To isolate cDNA clones which code for transcripts induced by DNA damaging agents in mammalian cells.

Methods Employed

Standard molecular biology techniques and specialized hybridization subtraction cloning approach which was developed in this laboratory.

Major Findings

50 different cDNA clones were isolated which code for different mRNA induced by UV radiation. These genes have been well conserved (which suggests important functions) since they were expressed and induced by UV in human cells. Several examples of abnormal expression were found in UV sensitive mutant cells. Many of these genes were also induced by other DNA damaging agents but not by unrelated injury such as heat shock.

Significance to Biomedical Research and the Program of the Institute

DNA damage and its repair play a central role in carcinogenesis and also in the cellular response to many antineoplastic agents. 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

A high priority is to determine the biologic importance of the sequences isolated. One approach is to study their expression in mutant cells as already described. The possibility exists that our clones may code for the mutated gene in these different cell lines. For fine mapping studies, full length cDNA clones need to be isolated. We plan to isolate full length clones from both a Chinese hamster and a human library. With the human clones, we can effectively study their expression in spontaneous mutants such as xeroderma pigmentosum. With full length clones, expression vectors can be constructed to determine if a particular sequence reverses the mutation in the mutant cell lines described above.

Publications

1. Fornace, A.J. Jr., Mitchell, J.B. Nucleic Acids Res. 14: 5793, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06381-01 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Modeling of Time-Dose Response of Human Tumors and Normal Tissues

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

PI:	J. van de Geijn	Radiation Physicist	ROB, NCI
Others:	J. Mitchell	Radiobiologist	ROB, NCI
	R. Miller	Radiation Physicist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation 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.)*

External beam therapy is generally given over many sessions, spread-out over several weeks. Although certain rules as to number of fractions, total time, and dose per fraction have evolved empirically over several decades of clinical experience, these rules are ill-understood. The present project attempts to develop a simple mathematical description of the time course of the reactions of both normal tissue and tumor, as a function of these variables. The limiting factor for normal tissue is its short-term and long-term minimum acceptable functionality. The latter is assumed to be determined by the balance between attrition of the functioning cells and the production of replacement cells generated by the residual viable stem cells, whose population is highly radiation sensitive. Local tumor cure requires incapacitating every clonogenic cell in the tumor.

GPO 914-918

The present method uses either of two models for single-dose response of stem cells and clonogenic tumor cells combined with certain concepts as to inter-fraction and post-treatment behavior of the normal tissues and any residual tumor. The various characteristics are adjusted empirically to obtain computed time-dose patterns of reactions similar to those observed clinically.

Project Description

Objectives: To develop a mathematical formalism describing:

1. The attrition of functioning normal tissue cells.
2. The survival rate, per single dose, of viable stem cells.
3. The inter-fraction and post-treatment course repopulation, after completion of the sub-lethal damage repair, of the viable stem cells.
4. The survival rate of clonogenic tumor cells per single-dose.
5. The inter-fraction and post-treatment growth pattern of the clonogenic cells as well as the gross tumor.

Methods Employed

1. The alpha/beta (2-parameter) model as well as the single-hit/ single-target, single-hit/multi-target (3-parameter) model are explored for the single-dose response of stem cells and clonogenic tumor cells.
2. Linear attrition over time is assumed for functioning normal tissue cells as well as non-clonogenic tumor cells.
3. Normal tissue cell loss and replacement is under homeostatic control.
4. Clonogenic tumor cells proliferate exponentially over time.
5. Stem-cell proliferation is triggered only after some distress signal related to functioning cell levels drop below a certain threshold.
6. Normal tissue tolerance is interpreted as the lower limit of normal tissue functionality: the normal tissue functioning cells dropping below some fraction of ther normal count.
7. P % tumor cure probability is assumed to be reached if at the end of treatment there are, statistically, 100-P clonogenic cells left per100 tumors.

Major Finding

1. A provisional mathematical model has been developed.
2. An interactive computer program has been developed which enables automatic search for acceptable parameters, based on estimates of reasonable ranges of certain key parameters, such as Do, cell doubling times, etc.
3. It is already possible to simulate time-dose response patterns for certain conventional and unconventional fractionation schemes, which are reasonably consistent with published findings in some clinical trials.

Significance to Biomedical Research and the Program of the Institute

1. The present model shows promise as a tool toward understanding of time-dose response to conventional or "standard" treatment schedules, as well as some hyper-fractionation schemes and other non-standard schemes.

2. The model might be used to explore, by simulation, other unconventional schemes. There is some reason to believe that, at least in cases where the reactions fast-renewing normal tisssues are the critical issue, new regimes might be worth investigating. This might shed light on a possible critical lower limit of quasi-instantaneous stem cell population, which might still allow sufficient early repopulation to prevent intolerably severe early functionality damage.

Proposed Course

1. Continuation of study of literature data.
2. Design of relevant experiments.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06382-01 RO

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Radiation Therapy with Radiolabelled Antibodies: Technical Aspects and Dosimetry

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

PI:	R. Miller	Radiation Physicist	ROB, NCI
Others:	A. Raubitschek	Radiotherapist	ROB, NCI
	J. van de Geijn	Radiation Physicist	ROB, NCI
	K. Orr-Yeakel	Dosimetrist	ROB, NCI

COOPERATING UNITS *(if any)*

Nuclear Medicine Department, CC; Diagnostic Radiology Department, CC

LAB/BRANCH

Radiation Oncology Branch

SECTION

Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.0	8.5	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.)*

Administration of radiolabelled antibodies is a relatively new treatment modality for certain forms of cancer. Much of this field is developmental in nature. In particular, the dosimetry of tumor masses, especially at the microscopic level, is as yet unknown. The Radiation Physics and Computer Automation Section is actively assisting in the implementation of clinical protocols. Current research is in two major areas. Imaging of the organ-specific distribution patterns on a temporal basis is fundamental to the understanding of antibody kinetics and for large volume radiation dosimetry (at the total organ level). The ability to accurately localize biodistribution patterns using nuclear medicine imaging techniques and to accurately register these images with respect to other imaging modalities (CT or MRI) is essential for obtaining quantitative results. Additionally, the dosimetry of microscopic tumor masses is best approached through the use of computer modelling. The results, where possible, will be validated using quantitative autoradiography.

P-S 6040 (Rev. 1/84)

GPO 914-916

Project Description

Objectives: To localize the sites of retention of radiolabelled antibodies and to determine the deposition-retention kinetics as well as the clearance pathways. To determine normal organ radiation doses and tumor dose, if possible on a microscopic level for alpha, beta and gamma emitting radionuclides. To determine the optimum combination of imaging modalities for localization and to determine the lower limits of detection of tumor masses with external imaging devices.

Methods Employed

This project will use small animal models to determine the metabolic pathways of various antibodies and their deposition-retention-excretion kinetics. Phantom studies will be conducted to determine the optimum imaging modalities and their lower limits of detection. These will be confirmed using large animal models. Computer models for determining dose distributions on a microscopic level and for alpha emitting radionuclides will be developed and tested with animal models. Patients under treatment will be imaged, as appropriate and will be bioassayed using external counting techniques. Biopsies will be taken and used to validate metabolic and dosimetric models for each radiolabelled antibody.

Major Findings

Studies at other institutions indicate that therapy with radiolabelled antibodies offers little advantage over conventional forms of radiation therapy in the treatment of large tumor masses, due to the inhomogeneous distribution pattern of organ uptake. This results in large dose gradients within the treated site. Antibody therapy shows real promise, however, in the treatment of small tumor masses, ideally microscopic disease. The problem with this approach is that the size of these masses makes them difficult to localize using traditional nuclear medicine imaging techniques. It may be possible to image these masses by employing other imaging modalities, either singly or in combination. Also, the dose calculational formalism for distributed radionuclide sources (MIRD), may no longer be valid under these conditions, since the range of the particulate radiations may be greater than the tumor mass and may be inhomogeneous, as well. A new formalism will need to be developed for alpha emitting radionuclides, as their energy deposition pattern differs significantly from beta-gamma emitters.

Significance to Biomedical Research and the Program of the Institute

Radiolabelled antibodies are a new, exciting potential treatment modality. They offer the promise of selectively irradiating tumor masses, while delivering minimal radiation doses to normal tissues. This represents the ideal form of radiation therapy. It is possible that, for some forms of cancer, radiolabelled antibody therapy will supplant chemotherapy as the treatment of choice for microscopic disease.

Proposed Course

To be continued. Perform phantom studies on imaging devices and begin animal experiments upon completion of the ROB animal facility.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06383-01 RO

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development of an Improved Treatment Chair for Radiation Therapy

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

PI:	R. Miller	Radiation Physicist	ROB, NCI
Others:	A. Raubitschek	Radiotherapist	ROB, NCI
	F. Harrington	Biomed. Engineering Tech.	ROB, NCI
	J. van de Geijn	Radiation Physicist	ROB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Radiation Oncology Branch

SECTION
Radiation Physics and Computer Automation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

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

This project is intended to design a treatment chair which overcomes the inherent design limitations of commercially available chairs. This chair will function independently of the treatment couch so as to permit opposed field treatment in any orientation in an extended isocentric fashion (the center of rotation will be at a distance greater than the standard isocenter of the accelerator). This chair will be capable of accurate, reproducible rotation and translation in the lateral, longitudinal and vertical planes. If possible, the chair will function properly with a standard radiotherapy simulator to permit proper localization, immobilization and treatment planning.

The chair will be designed on the "tool holder" principal. That is, the chair will function as a platform, providing for the attachment of various additional devices which can be placed in such a manner so that they allow proper immobilization of the patient without restricting treatment delivery.

Project Description

Objectives: To develop an independent treatment chair to permit multiple-field radiation therapy at either standard or extended SSD.

Methods Employed

The Radiation Oncology Branch machine shop fabricates any chair components and accessories that are needed. Selected patients are placed in the chair for simulation and for their course of therapy. Any problems associated with immobilization and repositioning are analyzed on a daily basis and the necessary modifications are made.

Major Findings

The current version of the treatment chair permits opposed-field treatments and can be used with the existing simulator as well as any treatment unit. Treatment of some forms of cancer with the patient seated is advantageous. The current design of the chair is excessively limited by the stipulation that it operate with the simulator. The hydraulic vertical motion is imprecise and the positioning of the patient by hand is operationally difficult. The center of rotation of the chair is also at an undesirable location, which precludes its use as an isocentric device.

Significance to Biomedical Research and the Program of the Institute

Treatment of the mediastinum with the patient seated can minimize the amount of lung in the irradiated field, minimizing complications. A combined Waldeyer's/ mantle field treatment is possible in this position. Also, low dose rate mantle fields can be used by placing the chair at an extended SSD.

Proposed Course

Develop a special simulator that can be used with the chair. Redesign the base of the chair to provide isocentric rotation and a more precise vertical motion. Also provide lateral and longitudinal motions that are driven either manually or powered, as opposed to the free-sliding motion currently used.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 03800-17 SURG

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Surgical Consultants & Collaborative Research Involving Surgical Services at NIH

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

PI: S.A. Rosenberg	Chief of Surgery, NCI	SURG, NCI
Others: Entire Staff	Surgery Branch	SURG, NCI

COOPERATING UNITS *(if any)*

GD Aurbach (NIAMDD), JL Doppman (CC), E Glatstein (NCI), J Robbins (NIAMDD), L Liotta (NCI), RC Young (NCI), P Pizzo (NCI), J Gardner (NIAMDD)

LAB/BRANCH

Surgery Branch

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Investigators in the Surgery Branch of the National Cancer Institute are the general surgeons and general surgical consultants to the entire National Institutes of Health. In this role we see patients in primarily two capacities. Firstly, we see patients in consultation for all general surgical and specialty surgical problems except for the specialties of cardiac and orthopedic surgery. The Surgery Branch answers all emergency as well as elective surgical consultations and provides 24 hour coverage for surgical emergencies that may arise in the Clinical Center Hospital.

Secondly, the Surgery Branch collaborates in the procurement of tissues for studies required by other investigative units. The degree of involvement of the Surgery Branch in the planning and execution of these studies is variable. The Surgery Branch often plays an instrumental role in the design of these studies while in other collaborations, the Surgical Service merely provides tissues.

Approximately 40% of the clinical surgical effort of the Surgery Branch is devoted to these consultative and collaborative studies.

A complete listing of surgical procedures performed by the Surgery Branch is presented in Table I. Surgery performed by surgical consultants operating within the Surgery Branch is listed in Table II.

Over 1000 consultations were received last year from other NCI Branches as well as other NIH Institutes.

RT

SURGICAL SERVICES DEPARTMENT

ANNUAL STATISTICS

APRIL 1986 - MARCH 1987

TOTAL PROCEDURES	HOURS	INSTITUTES/OTHERS	TOTAL PROCEDURES	
335	1154.50	Ward (NCI)	161	Emergencies
978	2035.00	Consult (NCI)	207	Add-ons
121	209.75	Med. Br. (NCI)	471	Cancellations
1434	3399.25	TOTAL (NCI)	259	OPD's
			36	2WCSR
1434	3399.25	NCI	4	ICU-2J
324	1416.25	NHLBI	3	MICU-10D
173 1/2	783.75	NINCDS	21	Other Cath Lab & 2W
46	38.75	Med. Neuro	2	2W
75	140.25	NEI		
13	19.00	ENT		
3	4.00	ROB	2105	Total Cases
14 1/2	37.25	NIDR	5886.25	Total Hours
10 1/2	24.00	Orthopedics		
5	7.75	NICHD		
6 1/2	16.00	Other - Outside Consultants		

MONTHLY SUMMARY

January	176	Total Procedures	July	178	Total Procedures
	524.00	Total Hours		555.25	Total Hours
February	187	Total Procedures	August	174	Total Procedures
	508.00	Total Hours		486.00	Total Hours
March	171	Total Procedures	September	187	Total Procedures
	444.00	Total Hours		497.75	Total Hours
April	185	Total Procedures	October	176	Total Procedures
	571.00	Total Hours		459.00	Total Hours
May	165	Total Procedures	November	157	Total Procedures
	445.50	Total Hours		434.00	Total Hours
June	181	Total Procedures	December	168	Total Procedures
	491.50	Total Hours		470.25	Total Hours

END

1. Udelsman, R., Roth, J.A., Lees, D., Jelenich, S.E., and Pass, H.I.: Endobronchial metastases from soft tissue sarcoma. J. Surg. Oncol. 32: 145-149, 1986.

2. Norton, J.A., Shawker, T.H., Jones, B.L., Spiegel, A.M., Marx, S.J., Fitzpatrick, L., Aurbach, G.D., and Doppman, J.L.: Intraoperative ultrasound and reoperative parathyroid surgery: An initial evaluation. World J. Surg. 10: 631-639, 1986.

3. Papa, M.Z., Shiloni, E., and McDonald, H.D.: Total colonic necrosis. A catastrophic complication of systemic lupus erythematosus. Dis. of the Colon & Rectum 29: 576-578, 1986.

4. Edington, H., Salwitz, J., Longo, D.L., Roth, J.A., and Pass, H.: Thymic hyperplasia masquerading as recurrent Hodgkin's disease: Case report and review of the literature. J. Surg. Oncol. 33: 120-123, 1986.

5. Norton, J.A., Doppman, J.L., Collen, M.J., Harmon, J.W., Maton, P.N., Gardner, J.D., and Jensen, R.T.: Prospective study of gastrinoma localization and resection in patients with Zollinger-Ellison Syndrome. Ann. Surg. 204: 468-479, 1986.

6. Lotze, M.T., and Wanebo, H.J.: Current and future research directions in management of hepatic cancer. In Wanebo, H. (Ed.): Hepatic and Biliary Cancer. New York, NY, Marcel Dekker, Inc., 1987, 501 pp.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM03801-17 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Clinical Studies in Cancer Surgery

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

PI: S.A. Rosenberg Chief of Surgery, NCI SURG, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

CHECK APPROPRIATE BOX(ES)

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

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

The Surgery Branch has a variety of studies investigating innovative therapies for patients with malignant diseases. The major emphasis of these studies is in the treatment of soft tissue sarcomas, osteogenic sarcomas, colorectal cancer, gastric cancer, renal cell cancer and melanoma. The major emphasis in Surgery Branch cancer therapy is in adjuvant therapy with emphasis on the use of combined treatment modalities in addition to surgery.

RT

PUBLICATIONS

1. Lawrence, W.T., Talbot, T.L. and Norton, J.A.: Preoperative or postoperative doxorubicin hydrochloride (Adriamycin): Which is better for wound healing? Surgery 100: 9-12, 1986.

2. Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Vetto, J.T., Seipp, C.A. and Simpson, C.: A new approach to the therapy of cancer based on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2. Surgery 100: 262-271, 1986.

3. Deutsch, H.L., Edington, H.D., McCullough, C.M., and Sugarbaker, P.H.: Prolonged skin allograft survival through enhancement effects on donor tissue. Transplantation 42: 200-204, 1986.

4. Sugarbaker, P.H., Gianola, F.J., Barofsky, I., Hancock, S.L. and Wesley, R.: 5-Fluorouracil chemotherapy and pelvic radiation in the treatment of large bowel cancer. Decreased toxicity in combined treatment with 5-fluorouracil administration through the intraperitoneal route. Cancer 58: 826-831, 1986.

5. Roth, J.A., Pass, H.I., Wesley, M.N., White, D., Putnam, J.B. and Seipp, C.: Comparison of median sternotomy and thoracotomy for resection of pulmonary metastases in patients with adult soft-tissue sarcomas. Ann. Thorac. Surg. 42: 134-138, 1986.

6. Chin, J.K., Rong, G.H., Scharff, J.E. and Sindelar, W.F.: Gastrointestinal carcinoma-associated antigen defined by a murine monoclonal antibody. J. Natl. Cancer Inst. 77: 599-604, 1986.

7. Lotze, M.T. and Rosenberg, S.A.: Protocol design for lymphokine testing in clinical studies of human cancer. Lymphokine Res. 5: S177-S181, 1986.

8. Sugarbaker, P.H.: The management of recurrent colorectal cancer. Intl. J. Colorec. Dis. 1: 133-151, 1986.

9. Sugarbaker, P.H. and Leighton, S.B.: Hepatic parenchymal suction dissector. Surg., Gynec., Obstet. 163: 267-269, 1986.

10. Lotze, M.T., Carrasquillo, J.A., Weinstein, J.N., Bryant, G.J., Perentesis, P., Reynolds, J.C., Matis, L.A., Eger, R.R., Keenan, A.M., Helstrom, I., Hellstrom, K.E. and Larson, S.M.: Monoclonal antibody imaging of human melanoma. Radioimmunodetection by subcutaneous or systemic injection. Ann. Surg. 204: 223-235, 1986.

11. Lotze, M.T. and Rosenberg, S.A.: Results of clinical trials with administration of interleukin 2 and adoptive immunotherapy with activated cells in patients with cancer. Immunobiol. 172: 420-437, 1986.

END

12. Gianola, F.J., Sugarbaker, P.H., Barofsky, I., White, D.E., and Meyers, C.E.: Toxicity studies of adjuvant intravenous versus intraperitoneal 5-FU in patients with advanced primary colon or rectal cancer. J. Clin. Oncol. 9: 403-410, 1986.

13. Sindelar, W.F., Hoekstra, H., Restrepo, C. and Kinsella, T.J.: Pathological tissue changes following intraoperative radiotherapy. J. Clin. Oncol. 9: 504-509, 1986.

14. Moley, J.F., August, D., Norton, J.A. and Sugarbaker, P.H.: Home parenteral nutrition for patients with advanced intraperitoneal cancers and gastrointestinal dysfunction. J. Surg. Oncol. 33: 186-189, 1986.

15. Seipp, C.A., Simpson, C. and Rosenberg, S.A.: Clinical trials with IL-2. Oncol. Nursing Forum 13: 25-29, 1986.

16. Lotze, M.T., Matory, Y.L., Rayner, A.A., Ettinghausen, S.E., Vetto, J.T., Seipp, C.A. and Rosenberg, S.A.: Clinical effects and toxicity of interleukin-2 in patients with cancer. Cancer 58: 2764-2772, 1986.

17. Edington, H.D., Hancock, S., Coe, F.L., and Sugarbaker, P.H.: Preliminary report of a new treatment strategy for advanced pelvic malignancy: Surgical resection and radiation therapy using afterloading catheters plus an inflatable displacement prosthesis in the treatment of advanced primary and recurrent rectal cancer. Surgery 100: 494-498, 1986.

18. Ettinghausen, S.E., Bonow, R.O., Palmeri, S.T., Seipp, C.A., Steinberg, S.M., White, D.E. and Rosenberg, S.A.: Prospective study of cardiomyopathy induced by adjuvant doxorubicin therapy in patients with soft tissue sarcomas. Arch. Surg. 121: 1445-1451, 1986.

19. Muul, L.M., Spiess, P.J., Director, E.P. and Rosenberg, S.A.: Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. J. Immunol. 138: 989-995, 1987.

20. Topalian, S.L., Muul, L.M. and Rosenberg, S.A.: Growth and immunologic characteristics of lymphocytes infiltrating human tumors. Surg. Forum 37: 390-391, 1987.

21. Peacock, J.L., Inculet, R.I., Corsey, R. and Norton, J.A.: Resting energy expenditure and body cell mass alterations in sarcoma patients. Surg. Forum 37: 11-13, 1987.

22. Belldegrun, A., Linehan, W.M., Robertson, C.N., and Rosenberg, S.A.: Isolation and characterization of lymphocytes infiltrating human renal cell cancer: Possible application for therapeutic adoptive immunotherapy. Surg. Forum 37: 671-673, 1987.

23. Vetto, J.T., Papa, M.Z., Lotze, M.T., Chang, A.E. and Rosenberg, S.A.: Reduction of toxicity of interleukin-2 and lymphokine-activated killer cells in humans by the administration of corticosteroids. J. Clin. Oncol. 5: 496-503, 1987.

24. Sindelar, W.F. and Kinsella, T.J.: Intraoperative radiotherapy in carcinoma of the stomach. In Magenkarzinom. Gall, F.P. (Ed.). Fortschritte in der Chirurgie, Band 3. W. Zuckschwerdt Verlag. Munich. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 03811-13 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Immunotherapy of Animal and Human Sarcomas

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

PI: S.A. Rosenberg Chief of Surgery, NCI SURG, NCI

Others: L. Muul (Expert), J. Mule (Staff Fellow), S. Schwarz (Biologist), P. Spiess (Biologist), S. Ettinghausen (Medical Staff Fellow), E. Director (Microbiologist), C. Hyatt (Biologist), D. Slavin (Biologist), K. Burchenal (Biologist), Alan Lefor (Medical Staff Fellow), B. Weiss (Guest Researcher), Arie Belldegrun (Visiting Scientist), Raj Puri (Visiting Associate), Avi Eisenthal (Visiting Associate), James Yang (Senior Investigator)

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	6	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.)*

Attempts are being made to develop adoptive immunotherapeutic techniques utilizing the transfer of cells grown in long-term culture in interleukin-2. Techniques for the prolonged growth of cytotoxic and proliferative T cell lines and clones with anti-tumor reactivity have been developed. These cells have been shown to mediate the immunologic rejection of allografts and syngeneic tumors and attempts to use these cells in the adoptive immunotherapy of mouse and human tumors are in progress. A new class of cytotoxic cells has been described in both the mouse and the human. These lymphokine activated killer (LAK) cells develop selective cytotoxicity for cancer cells following incubation in the lymphokine, interleukin-2. The adoptive transfer of these cells into mice bearing established tumors can mediate the inhibition of pulmonary and hepatic metastases. The systemic administration of interleukin-2 has been shown to enhance immune responses in vivo.

In the past year, immunotherapeutic trials studying the effects of adoptive transfer of lymphokine activated killer cells and recombinant IL-2 into patients with advanced cancer have continued and new adjuvant trials begun. Clinical trials with tumor infiltrating lymphocytes have also begun.

1. Shu, S. and Rosenberg, S.A.: Adoptive immunotherapy of a newly induced sarcoma: Immunologic characteristics of effector cells. J. Immunol. 135:2895-2903, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06654-10 SURG

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Studies in Malignant Disease

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

P.I. W. F. Sindelar Senior Investigator SURG NCI

COOPERATING UNITS *(if any)*

Others: T. Kinsella Senior Investigator SURG NCI

LAB/BRANCH

Surgery Branch

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	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.)*

Patients with gastrointestinal carcinomas have been studied for evidence of reactivity against tumor-associated determinants expressed on both fresh and cultured syngeneic or allogeneic tumor cells using immunoperoxidase staining techniques. Tumor-associated antigens have been isolated from both animal and human pancreatic cancers and have been investigated for possible applications to immunotherapy or methods of immunodiagnosis. Monoclonal antibodies have been developed to tumor-associated determinants in both hamster and human pancreatic cancers and have been demonstrated to be cytotoxic cells both in vitro and in vivo. A clinical trial evaluating monoclonal antibody therapy in advanced pancreatic cancer patients produced some clinical responses. Tolerance of various normal and surgically-manipulated tissues to intraoperative radiotherapy has been investigated in dogs to determine both acute and long-term toxicity from radiation effects.

GPO 914-916

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06657-05 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies in Cancer Cachexia and Zollinger Ellison Syndrome (ZES)

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

P.I. J.A. Norton, M.D. SURG, NCI

Others: K. Kern, M.D. MSF
D. Cromack, M.D. MSF
D. Fraker, M.D. MSF
M. Stovroff, M.D. MSF
G. Darling, M.D. Expert SURG, NCI
C. Gorschboth, Technician

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Surgery Branch

SECTION
Surgical Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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.)*

Cancer Cachexia

Resting energy expenditure is elevated and body cell mass (BCM) is significantly reduced in noncachectic sarcoma patients[1]. Whole body leucine turnover and protein synthesis is also elevated in these patients[2]. BCM is reduced in weight losing cancer patients[3].

Rat[4] and human[5] sarcomas have increased uptake of deoxyglucose which allows them to be imaged. In humans, uptake of labelled deoxyglucose correlated with sarcoma grade and prognosis[5].

Insulin is a potent anti-cachexia hormone. In experiments in rats, it overcomes the toxic effects of cachectin[6]. It reverses the toxicity of doxorubicin and improves the anti-tumor effects of doxorubicin[7]. It appears to shift glucose and amino acids from the tumor to the host[8].

Cachectin may be a mediator of cancer cachexia. Rats who develop tolerance to ip cachectin survive longer following a tumor challenge than controls[9].

Zollinger Ellison Syndrome (ZES)

Portal venous sampling (PVS) is sensitive but not specific for localizing gastrinomas[10]. Computed tomography (CT) is less sensitive than PVS but more specific[11]. Selective arteriography is the best study to preoperatively localize gastrinomas. It has a sensitivity of 80% and a specificity of 100%[12]. Using a strategy of medical control of the gastric acid hypersecretion and aggressive surgical resection of gastrinomas in 32 patients with ZES, 30% were biochemically and radiographically NED with a mean followup of 2 years postoperatively[13].

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM06658-05 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of the Pineal Gland Hormone Melatonin and Estrogen Receptor Activity

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

D. N. Danforth, Jr., M.D., Senior Investigator — SURG NCI

Others: B. Gabriel, Visting Fellow — SURG NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Surgery Branch

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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.)*

We are studying the effect of the pineal gland hormone melatonin on the metabolism of human breast cancer cells. We have shown that melatonin rapidly enhances protein synthesis in human breast cancer cells. Pulse chase experiments of the melatonin-induced protein fraction demonstrates that this fraction translocates from the cytoplasm to the nucleus. Polyacrylamide electrophoresis of the cytoplasm of melatonin treated cells shows the presence of a 3,000 Dalton polypeptide in the melatonin but not vehicle treated cells. Translocation to the nucleus is associated with enhanced RNA synthesis. The RNA fraction which is enhanced has been shown to be ribosomal RNA. We are currently purifying and characterizing the melatonininduced polypeptide, and analyzing the effect of enhanced ribosomal RNA synthesis on growth and metabolism of these cells.

We have also characterized the large molecular weight forms of the estrogen receptor in human breast cancer cells. We have shown that the ER is present as a 320 K and 600 K large molecular weight protein which is stabilized by protein inhibitors and not dissociated by high salt conditions. Studies are underway to further characterize these large molecular weight forms and to determine their significance in patients with breast cancer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06659-05 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

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

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

P.I.	W. M. Linehan, Head, Urologic Oncology Section		SURG, NCI
Others:	C.N. Robertson, M.D.	Expert	SURG, NCI
	Len Gomella, M.D.	Medical Staff Fellow	SURG, NCI
	Patrick Anglard, Ph.D.	Visiting Fellow	SURG, NCI
	Eric Sargent, M.D.	Guest Researcher	SURG, NCI
	Beth Miller, B.S.	Microbiologist	SURG, NCI
	Emil Trahan, B.S.	Biochemist	SURG, NCI

COOPERATING UNITS *(if any)*
Others: Dr. B. Zbar - NCI
Dr. M. Israel - NCI

LAB/BRANCH
Surgery Branch

SECTION
Urologic Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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

We are studying the molecular genetics of renal cell carcinoma, evaluating growth factor production by genitourinary tumors and participating in studies of adoptive immunotherapy in patients with advanced malignancies. In collaboration with Berton Zbar we tested by the molecular technique of restriction fragment polymorphism analysis for a DNA sequence deletion in the short arm of chromosome 3 in both neoplastic and somatic tissue in patients with sporadic renal cell carcinoma. We found loss of alleles at loci in the short arm of chromosome 3 in all 11 of the patients who could be evaluated. We also tested for gene loss at chromosome 11 and 15 and found no evidence for a generalized reduction to homozygosity. Loss of specific gene products from somatic cells may be critical in the origin or evolution of renal cell carcinoma and suggest that a recessive oncogene may be involved in tumorogenesis. Studies are in progress to analyze by RFLP analysis more pairs in somatic tissue, to analyze metastatic tissue for gene deletion and to further characterize possible gene deletions at other chromosomal sites. Other collaborative studies to further characterize the area of DNA deletion and to introduce a nondeleted chromosome 3 into renal cell carcinoma are underway. We have demonstrated that tumor necrosis factor stimulates bone resorption and that it does so in a hormone-like fashion and that prostate carcinoma produces a factor which stimulates bone resorption in a transforming growth factor-like fashion. We have evaluated patients with metastatic and locally advanced renal cell carcinoma prior to entry into therapy with adoptive immunotherapy and have performed over 40 radical nephrectomies in the last two years in patients with renal cell carcinoma as part of this protocol and in support of basic and clinical studies of tumor infiltrating lymphocytes. We have also characterized the renal toxicity in patients treated with Interleukin-2, which is a major dose-limiting aspect to this therapy.

1. Robertson, C.N., Santora, A, Liang, C.C., Linehan, W.M.: Human recombinant TNF mediates bone resorption in vitro. Surg. Forum 37:669-670, 1986.

2. Belldegrun, A., Linehan, W.M., Robertson, C.N., Rosenberg, S.A.: Isolation and characterization of lymphocytes infiltrating human renal cell cancer: Possible application for therapeutic adoptive immunotherapy. Surg. Forum 37:671-673, 1986.

3. Linehan, W.M. and Andriole, G.L.: Endocrine Aspects of Urologic Oncology. In Graham, S.D. (Ed.). Urologic Oncology, New York, Raven Press, pp 75, 1986.

4. Andriole, G., Macher, A., Reichert, C., Mazur, H., Gelman, E., Linehan, W.M.: Aids, case for diagnosis. Military Medicine, 151:M49-M56, 1986.

5. Rosenberg, S.A., Lotze, M.T., Muul, L.M., Chang, A.E., Avis, F.P., Leitman, S., Linehan, W.M., Robertson, C.N., Lee, R.E., Rubin, J.T., Seipp, C.A., Simpson, C.G., and White, D.E.: Clinical experience with the treatment of 157 patients with advanced cancer using lymphokine activated killer cells and interleukin-2 or high dose interleukin-2 alone. N. Eng. J. Med. 316: 889-897, 1987.

6. Helman, L.J., Thiele, C.J., Linehan, W.M., Baylin, S.B., and Israel, M.A.: Molecular markers of neuroendocrine development and evidence of environmental regulation. Proc. Natl. Acad. Sci. U.S.A. 84:2336-2339, 1987.

7. Udelsman, R., Norton, J.A., Jelenich, S.E., Goldstein, D.S., Linehan, W.M., Loriaux, D.L., Chrousos, G.P.: Hormone responses during neck exploration: Hypothalamic-pituitary-adrenal, reninangiotensin and sympathetic system responses. J. Clin. Endocrinol. and Metabolism 64(5):986-994, 1987.

8. Andriole, G.L., Rittmaster, R.S., Loriaux, D.L., Kish, M.L., Linehan, W.M.: The effect of 4MA, a potent inhibitor of 5 alpha-reductase, on the growth of PC-82, a human, androgen-dependent prostatic cancer. Prostate, 10(3):189-197, 1987.

9. Belldegrun, A., Linehan, W.M., Topalian, S., Robertson, C.N. and Rosenberg, S.A.: Growth and clonal analysis of tumor infiltrating lymphocytes and tumor involved draining lymph node cells from patients with renal cell cancer. Surgical Forum 1987 (in press).

10. Andriole, G., Macher, A., Reichert, C., Mazur, H., Gelman, E., Linehan, W.M.: Case 7. In John C. Duffy (Ed.): Registry of Aids Pathology. Baltimore, Williams & Wilkins, 1987 (in press).

11. Robertson, C.N. and Linehan, W.M.: Kidney cancer, new and potential approaches to therapy. In Magrath, I.T. (Ed.): New and Potential Approaches to Cancer Therapy, Springer Verlag, (in press).

12. Linehan, W.M.: Rhabdomyosarcoma of the Prostate. In Resnick, M.I. and Kursh, E.D. (Eds.). Current Therapy in Genitourinary Surgery, Philadelphia, B. C. Decker Company, (in press).

13. Zbar, B., Brauch, H., Talmadge, C., and Linehan, M.: Loss of genes on the short arm of chromosome 3 in renal cell carcinoma. Nature (in press).

14. Belldegrun, A., Webb, D.E., Austin, H.A., Steinberg, S.M., White, D.E., Linehan, W.M., and Rosenberg, S.A.: Effects of Interleukin-2 on renal function in patients receiving immunotherapy for advanced cancer. Ann. Int. Med. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT .

PROJECT NUMBER

Z01 CM 06660-04 SURG

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

The Study of Specific Adoptive Immunotherapy

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

PI:	A. E. Chang	Senior Investigator	SURG, NCI
Others:	D. Perry-Lalley	Microbiologist	SURG, NCI
	B. Ward	Medical Staff Fellow	SURG, NCI
	K. Stephenson	Medical Staff Fellow	SURG, NCI
	K. Griffith	Medical Staff Fellow	SURG, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

Tumor Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	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.)*

This laboratory is involved in developing methods to generate lymphoid cells which confer specific antitumor activity upon adoptive transfer. Murine models involving weakly immunogenic and non-immunogenic tumors have been utilized to examine the mechanisms involved in tumor immunization and tumor rejection. Utilizing monoclonal antibodies which can recognize subsets of cells has enabled us to define the role of $L3T4^+$, $Lyt\text{-}2^+$ and Ia^+ cells in tumor rejection. Selective expansion of lymphoid cells in vitro with anti-tumor reactivity in vivo can be accomplished by incubation with interleukin-2 (IL-2) and secondary stimulation with irradiated tumor. This methodology is being employed to investigate the generation of lymphoid cells from tumor-bearing hosts. Efforts in treating hosts bearing established advanced macroscopic tumor is also being pursued.

PUBLICATIONS

1. Chang, A.E., Shu, S., Chou, T., Lafreniere, R., and Rosenberg, S.A.: Differences in the effects of host suppression on the adoptive immunotherapy of subcutaneous and visceral tumors. Cancer Res. 46:3426-3430, 1986.

2. Ward, B.A., Chou, T., Chang, A.E., Shu, S., and Rosenberg, S.A.: Specific adoptive immunotherapy: Characterization of lymphoid subpopulations involved in tumor rejection. Surg. Forum 37:416-418, 1986.

3. Ward, B.A., Perry-Lalley, D., Shu, S., and Chang, A.E.: Ia^+ cell participation in specific adoptive immunotherapy. Surg. Forum (in press).

4. Stephenson, K.R., Perry-Lalley, D., Shu, S., and Chang, A.E.: The role of regional lymph nodes in the development of tumor immunity. Surg. Forum (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT .

PROJECT NUMBER

Z01 CM 06661-04 SURG

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Immunologic Studies in Patients with Cancer

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

PI:	M.T. Lotze	Senior Investigator	SURG, NCI
Others:	M.C. Custer, B.S.	Microbiologist	SURG, NCI
	E. Weibke, M.D.	Medical Staff Fellow	SURG, NCI
	S. Tomita, M.D., Ph.D.	Visiting Associate	SURG, NCI
	D. Jablons, M.D.	Medical Staff Fellow	SURG, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our laboratory's major work is the development and evaluation of immunologic reagents in patients with malignancy. Preparation of single cell suspension from human tumors and derivation of cloned and bulk populations of autologous lymphocytes reacting to them remain a major goal. In the last year, over 100 tumor preparations have been evaluated and currently attempts to grow tumor reactive cells directly from tumors is being evaluated. We have been able to clone lymphoid cells directly from human tumors (TILs) with reactivity unique to autologous melanoma. In addition we are evaluating new technologies for the early separation and growth of TILs. We have evaluated the susceptibility of tumor cells to lysis by TILs as well as cells with NK and lymphokine activated killer (LAK) activity. We have demonstrated that 100-1000 fold increase in tumor susceptibility to lysis by some of these reagents may occur with pre-incubation of the targets with gamma interferon (γ-IFN) and tumor necrosis factor (TNF), two recently cloned recombinant human cytokines. We have investigated the in vivo use of Interleukin-2 (IL-2) in patients and reported in detail the responses seen in patients receiving IL-2 IV or IP. Both partial and complete responses have been noted in patients with renal cell tumors as well as melanoma. Future effects include therapeutic application of cloned TILs and development of protocols for pretreatment of the host with TNF and γ-IFN on immunotherapy protocols.

Investigation of the use of monoclonal antibodies in conjunction with IL-2 or adoptive therapy with IL-2 activated cells has been carried out in our laboratory. We have evaluated heteroconjugated antibodies as well as antibodies to melanoma given alone as diagnostic agents or in conjunction with IL-2. Future efforts include a protocol designed to administer IL-2 at high doses in conjunction with therapeutic doses of monoclonal antibodies to patients with colorectal tumors as well as melanoma.

1. Perez, P., Titus, J.A., Lotze, M.T., Cuttita, F., Longo, D.L., Groves, E.S., Rabin, H., Durda, P.J. and Segal, D.M.: Specific lysis of human tumor cells by T cells coated with anti-T3 crosslinked to anti-tumor antibody. J. Immunol. 137:2069-2072, 1986.

2. Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Vetto, J.T., Seipp, C.A. and Simpson, C.A.: A new approach to the therapy of cancer based on the systemic administration of autologous lymphokine activated killer cells and recombinant interleukin-2. Surgery 100:261-271, 1986.

3. Lotze, M.T., Chang, A.E., Seipp, C.A., Simpson, C., Vetto, J.T. and Rosenberg, S.A.: High dose recombinant interleukin-2 in the treatment of patients with disseminated cancer: Responses, treatment related morbidity and histologic findings. JAMA. 256: 3117-3124, 1986.

4. Lotze, M.T. and Rosenberg, S.A.: Results of clinical trials with the administration of interleukin-2 and adoptive immunotherapy with activated cells in patients with cancer. Immunobiology. 172: 420-437, 1986.

5. Lotze, M.T. and Rosenberg, S.A.: Protocol design for lymphokine testing in clinical studies of human cancer. Lymphokine Res. 5: S177-S182, 1986.

6. Lotze, M.T, Custer, M.C. and Rosenberg, S.A.: Intraperitoneal administration of interleukin-2 in patients with cancer. Arch. Surg. 121: 1373-1379, 1986.

7. Lotze, M.T., Carrasquillo, J.A., Weinstein J.N., Bryant, G.J., Perentesis, P., Reynolds, J.C., Matis, L.A., Eger, R.R., Keenan, A.M., Hellstrom, I., Hellstrom, K-E. and Larson, S.M.: Monoclonal antibody imaging of human melanoma: Radioimmunodetection by subcutaneous or systemic injection. Ann. Surg. 204:223-235, 1986.

8. Rosenberg, S.A., Lotze, M.T., Muul, L.M., Chang, A.E., Avis, F.P., Leitmann, S., Linehan, W.M., Robertson, C.N., Lee, R.E., Rubin, J.T., Seipp, C.A., Simpson, C.G. and White, D.E.: Clinical experience with the treatment of 157 patients with advanced cancer using lymphokine activated killer cells and interleukin 2 or high dose interluekin-2 alone. NEJM 316:889-905, 1987.

9. Vetto, J.T., Papa, M.Z., Lotze, M.T., Chang, A.E. and Rosenberg, S.A.: Reduction of toxicity of interleukin-2 and lymphokine activated killer cells in humans by the administration of corticosteroid. J. Clin. Oncol. (in press).

10. Roberts, K., Lotze, M.T. and Rosenberg, S.A.: The lymphokine activated killer cell. I. Separation and functional studies of the human precursor and effector cell. Cancer Research (in press).

11. Gaspari, A., Lotze, M.T., Rosenberg, S.A., Stern, J.B. and Katz, S.I.: Cutaneous changes associated with interleukin-2 administration; Development of erytheoderma and cutaneous lymphocytic infiltration. JAMA (in press).

12. Lotze, M.T., Roberts, K., Custer, M.C., Segal, D.A. and Rosenberg, S.A.: Specific binding and lysis of human melanoma by IL-2 activated cells coated with anti-T3 and Anti-Fc receptor crosslinked to antimelanoma antibody: A possible approach to the immunotherapy of human tumors. J. Surg. Res. (in press).

13. Skibber, J.M., Lotze, M.T., Uppenkamp, I., Ross, W. and Rosenberg, S.A.: Identification and expansion of human lymphokine activated killer cells: Implications for the immunotherapy of cancer. J. Surg. Res. (in press).

14. Denicoff, K.D., Rubinow, D.R., Papa, M.Z., Simpson, C., Seipp, C.A., Lotze, M.T., Chang, A.E., Rosenstein, D. and Rosenberg, S.A.: The neuropsychiatric effects of interleukin 2/lymphokine activated killer cell treatment. Ann. Int. Med. (in press).

15. Lotze, M.T. Biology of IL-2 and rationale for its clinical application. In Rosenberg, S.A.: New approaches to the immunotherapy of cancer. Ann. Int. Med. (in press).

16. Lotze, M.T. and Rosenberg, S.A. IL-2 as a pharmacologic reagent. In Smith, K. (Ed.): Lymphokines. Academic Press, New York, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 06662-01 SURG

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of Phototherapy for Thoracic Malignancies

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

P.I.: H.I. Pass, M.D., Senior Investigator — SURG, NCI

Others: W.E. Rizzoni, M.D., Medical Staff Fellow — SURG, NCI
W. Matthews, B.S., Medical Technician — SURG, NCI

COOPERATING UNITS *(if any)*
Radiation Oncology Branch, NCI (A. Russo, M.D., Ph.D., J. Mitchell, Ph.D.)

LAB/BRANCH
Surgery Branch

SECTION
Thoracic Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	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.)*

Our laboratory has investigted the use of photodynamic therapy (PDT) for the treatment of thoracic malignancies by sensitization of malignant cells with a porphyrinoid (HPD) followed by illumination with 630 nM light. The in vitro sensitivity of human lung cancer line A 549 has been studied revealing that the cytotoxicity is dependent on the amount of energy input as well as the sentizer concentration used. A dose-rate effect has been revealed when cells were treated with .09 mW/cm^2 vs. .27 mW/cm^2. Cells incubated longer with the sensitizer had a significantly less surviving fraction by colony formation assay. Differences in in vitro sensitivity correlated with degree of cellular HPD concentration as measured by fluorescence, the protein concentration of the cells, as well as the size of the cells. Use of a light scattering media (intralipid) was able to augment the PDT cytotoxicity despite decreases in cellular HPD. Animal models of pulmonary metastases, tracheal tumors, as well as subcutaneous models have been established using the F344 rat sarcoma for the testing in vivo PDT. In vitro PDT of the F 344 rat sarcoma cell line has revealed it also to be sensitive to PDT. Two patients with complete bronchial obstruction due to metastatic malignancies have been palliated with PDT with partial or total lung expansion in both cases, without morbidity.

ART — PUBLICATIONS

1. Rizzoni, W.E., Matthews, W., Mitchell, J., and Pass, H.I. In vitro photodynamic therapy of human lung cancer: influence of dose-rate, hematoporphyrin concentration and incubation time, and cellular targets. Surg. Forum 1987, in press.

END —

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09290-02 OAD

PERIOD COVERED

October 1, 1986 to September 30, 1987

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	Sr. Staff Fellow	OAD, NCI
Others:	M. Mercep	Visiting Fellow	OAD, NCI
	J. Sussman	Howard Hughes Medical Scholar	
	D. Hernandez	Biologist	OAD, NCI
	S. Bridges	Visiting Fellow	OAD, NCI
	D.L. Longo	Chief	OAD, NCI

COOPERATING UNITS *(if any)*

Lab. of Cellular and Molecular Biology, OBRR/CDB (P. Noguchi), Lab. of Immunol., NIAID (E. Shevach), Radiation Oncology Branch, NCI (A. Russo), Lab. of Immunol., NIAID (M. Jenkins and R. Schwartz), Immunol. Branch, NCI, (J. Bluestone).

LAB/BRANCH

Office of the Associate Director

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	.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.)*

We have demonstrated that the signal(s) transmitted upon occupancy of the antigen-specific receptor inhibit the growth of transformed T cells. This observation has been extended by using monoclonal antibodies to activate T cells. Within 24 hours of exposure to stimulatory antibodies directed against CD3 (mouse and human T cells) or Thy-1 (mouse T cells), transformed T cells secreted interleukin 2 and ceased their spontaneous proliferation. The specificity of this effect was ascertained in several ways: (1) external cross-linking of the anti-CD3 antibodies, either by Fc receptor-bearing accessory cells or by adherence to plastic, was required for growth to be inhibited, and (2) non-stimulatory anti-Thy-1 antibodies blocked the action of the stimulatory antibodies, i.e., allowed the transformed T cells to grow normally. The cell cycle block in T cell hybridomas, but not a chemically-induced transformed T cell, was followed by death, as evidenced by the release of 52-Cr or LDH. Cell cycle analysis demonstrated that the activated cells developed a block between the G1 and S phases, and the transit of cells in S phase was slowed. Furthermore, the chemically-induced T cell tumor, but not the T cell hybridoma, appeared to be able to assume a G0-like state, perhaps explaining its failure to rapidly lyse in response to activation. The in vivo growth of antigen-specific T cell hybridomas has been studied in a mouse tumor model. Introduction of the tumor (subcutaneously) and of the appropriate stimulatory antigen (intraperitoneally) resulted in complete rejection of the neoplasm. Even when treatment was not initiated until the tumor was well established, injection of antigen caused the tumor at the primary site of injection to regress in virtually all cases, resulted in long-term survival in over one-third of the animals. Studies using stimulatory monoclonal anti-CD3 or anti-Thy-1 antibodies in this tumor model system are planned.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Staff Fellow	OAD, NCI
Mladen Mercep	Visiting Fellow	OAD, NCI
Jeffrey Sussman	Howard Hughes Medical Scholar	
Diana Hernandez	Biologist	OAD, NCI
Sandra Bridges	Visiting Fellow	OAD, NCI
Dan L. Longo	Chief	OAD, NCI

OBJECTIVES

1. To study the mechanisms by which such activation events result in cell cycle block and growth inhibition of transformed T cells;

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

3. To explore in vivo models and develop methods of eliminating T cell neoplasms of unknown antigen-specificity from the tumor-bearing animal;

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

MAJOR FINDINGS

I. Activation-Associated Growth Inhibition of Transformed T Cells

We have previously demonstrated that activation of antigen-specific T cell hybridomas with the appropriate antigen/MHC combination results in lymphokine production and growth inhibition. Inhibition of growth was directly proportional to the degree of stimulation, and was a direct effect upon the cell, i.e., it was not mediated by the action of secreted lymphokines. Cell cycle analysis determined that antigen-mediated activation induced a G_1/S block. The effects of other T cell stimulatory signals on the growth of transformed T cells have now been explored. First, anti-CD3 antibodies, such as 2C11 (mouse T cells) and OKT3 (human T cells) have been evaluated for their ability to mimic the effects of antigenic stimulation. In the presence of Fc receptor-bearing accessory cells, or when cross-linked to a plastic surface, 2C11 induced interleukin 2 (IL-2) production from both T cell hybridomas and the chemically-induced T cell lymphoma, EL-4. IL-2 production was accompanied by a reciprocal decrease in T cell proliferation. This phenomenon was not restricted to murine T cells, as evidenced by the similar effects of OKT3 upon the human leukemia/lymphoma designated Jurkat. Furthermore, the phenomenon of growth inhibition was apparent when transformed T cell were activated by mitogenic antibodies directed against molecules not physically associated with the CD3/Ti T cell antigen receptor complex. For example, the monoclonal anti-Thy-1 antibody termed G7 was found to inhibit the growth of T cell hybridomas and EL-4. Cell cycle analysis found that G7 caused a G_1/S cell cycle block, as well as a block of cells in early S phase. With EL-4 it appeared that a portion of the

cells assumed a G_0-like state, evidenced by a decrease in the size of cells with the DNA content of G_1 cells. Non-stimulatory anti-Thy-1 monoclonal antibodies had no effect on transformed T cell growth; in fact, cross-blocking non-stimulatory anti-Thy-1 antibodies abrogated the effects of G7. The mitogenic anti-Ly-6 antibody, D7, was also found to cause transformed T cells to produce IL-2 and cease proliferating. Finally, activation with either G7 or 2C11 was found to induce the rapid lysis of T cell hybridomas, as evidenced by the release of ^{51}Cr or LDH. Preliminary experiments with CD3/Ti variants that no longer expressed the T cell antigen receptor suggested that anti-Thy-1 mediated signalling for growth inhibition requires the co-expression of CD3. Currently, efforts are underway to establish the validity of this result, and to determine which second signals, such as phosphatidylinositol hydrolysis or increases in intracellular free Ca^{2+}, are necessary to cause growth inhibition.

II. Activation-Induced Growth Inhibition as a Means of Treating T Cell Tumors In Vivo.

The ability of antigenic stimulation to inhibit the growth of transformed T cell in vitro prompted us to investigate its effect upon the growth of transformed T cell in vivo. To this end, antigen-specific T cell hybridomas were injected subcutaneously into syngeneic mice. The control group was treated with 1 mg of an irrelevant protein i.p. every other day for 8 treatments, while the experimental group received 1 mg of the appropriate stimulatory antigen, such as pigeon cytochrome c or hen egg lysozyme, according to the same protocol. When therapy was initiated on the same day that the tumors were inoculated, treatment with antigen resulted in virtually 100% "cure", as evidenced by (1) the failure of tumors to form at the site of inoculation and (2) long-term survival of the mice. Then from 7 to 11 days were allowed to elapse prior to the initiation of therapy, treatment with antigen resulted in remission of the primary tumor (average area of from 1 to 2.5 cm^2) in virtually all animals. Many of these animals proceeded to die of metastatic disease, but almost 40% became long-term survivors and never exhibited any signs of recurrent disease.

The mechanism of tumor rejection was investigated in two ways. First, nude mice were injected with a T cell hybridoma and treated with antigen as above. In each of three independent experiments, the appearance of tumors was substantially delayed, i.e., from 9 days in controls to 24 days in treated, by administration of the appropriate antigen. However, unlike in normal mice, the majority of nude mice did develop tumors despite treatment with antigen. That T hybridoma cell activation was necessary for any therapeutic benefit was demonstrated by the finding that tumors grown in nude mice that were MHC-incompatible with the T cell tumors, i.e., that lacked the correct class II molecule to present the antigen to the T cells, failed to respond at all to treatment with antigen. Second, normal mice that had survived an initial inoculation with the T cell hybridoma because of antigenic treatment were reinoculated with the same, or a similar, T cell hybridoma from 2 to 8 weeks later. None of these mice developed a tumor, despite the fact that they received no further therapy. Together, these experiments suggested that host T cell immunity plays a role in the rejection of these T cell tumors. This was confirmed by experiments in which splenocytes from "cured" animals were transferred i.v. to lightly irradiated syngeneic mice. The recipients were then challenged with the T cell hybridoma as above, but received no antigen

therapy. The transfer of whole splenocytes, but not anti-Thy-1 plus complement depleted splenocytes, prevented tumors from forming.

This model system has shown that activation of T cell hybridomas in vivo can result in their elimination. In order to be useful, however, one would require a less specific reagent than antigen, because it is not possible to predict the antigen-specificity of random T cell tumors. Experiments utilizing stimulatory monoclonal antibodies to treat T cell tumors, such as the anti-CD3 antibody 2C11, are being initiated.

III. Radiation Sensitivity of Antigen Presentation

Resting (non-cycling) B cells are capable of presenting soluble antigens to T cells. We have shown, however, that in contrast to low density antigen-presenting cells (APC), the ability of resting B cells to present antigen is very radiosensitive. There are three major areas in which radiation might affect antigen-presentation: (1) expression of MHC-encoded class II molecules, (2) antigen uptake and processing, and (3) the provision of poorly-described "second signals" to the responding T cell. These possibilities have been explored. First, although radiation may impair antigen processing, this does not seem to be the major antigen-presentation defect. This is inferred from experiments in which intact antigen molecules or antigen fragments, which do not require processing, were used to stimulate normal T cell clones. Presentation of both types of antigen was radiosensitive, and does-response analyses demonstrated that both were impaired at the same doses of radiation. Furthermore, when B cell APCs that had been exposed to antigen (pulsed) for 2 hours prior to irradiation were compared to B cell APC that were irradiated prior to antigen pulsing, no significant difference was demonstrated in their radiosensitivity. The possible effect of radiation on class II molecule expression was examined in two ways. First, fluoresceinated antigen staining of B cells 4 hours after irradiation (when cell size and ability to exclude trypan blue are maximally affected) demonstrated no change in cell surface class II levels. Second, when irradiated B cells were used to stimulate antigen-specific normal T cell clones, it was found that rather than being activated, these T cells were unresponsive to subsequent challenge with antigen in the presence of low density APC. This result is similar to that observed when fixed low density APC were used to present antigen to T cell clones. In the case of irradiated B cells, the induction of unresponsiveness required that the correct antigen and class II molecules were found on the B cell APC surface, and although they did not proliferate, the T cells enlarged and secreted IL-3.

To determine if the induction of unresponsiveness was due to the failure to provide necessary "second signals" to the T cell, allogeneic cells were added back to the T cell/irradiated B cell culture at its initiation. This resulted in excellent antigen-dependent T cell stimulation. Allogeneic resting B cells could provide this restorative function, but low density APC were better. Irradiation of the added-back allogeneic B cells, or fixation (e.g. with paraformaldehyde) abrogated the ability of these cells to co-induce T cell activation. IL-1 could not restore the ability of irradiated B cells to activate T cells. Together, these results are compatible with the notion that T

cell activation requires the delivery of a signal(s) distinct from antigen receptor occupancy, and that this function is radiosensitive in B cell, but now low density, APC. The nature of this second signal, and how it might promote T cell activation, is being investigated.

SIGNIFICANCE

The finding that perturbation of certain cell surface molecules on transformed T cell leads to both activation (lymphokine production) and inhibition of growth is of interest on several levels. First, it suggests that the mechanisms that control their spontaneous growth are susceptible to modification or modulation by presumably normal intracellular signalling events. Second, it raises the question of what, if anything, distinguishes T lymphocytes in this regard, and are there unique intracellular pathways that govern this response. Third, it offers the possibility of manipulating the growth of neoplastic T cells in vivo. By using antibodies directed against nonclonally distributed and nonpolymorphic molecules such as CD3 or Thy-1, it may be possible to alter the neoplastic growth of transformed T cells whose antigen-specificity is unknown.

PUBLICATIONS

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Malek, T.R., Ashwell, J.D., Germain, R.N., Shevach, E.M., and Miller, J.: The murine interleukin-2 receptor: Biochemical structure and regulation of expression. Immunol. Rev. 92: 81-101, 1986.

Ashwell, J.D., Fox, B.S., and Schwartz, R.H.: Use of a receptor competition assay to explore the interaction of the T cell antigen-specific receptor with its ligands. Fed. Proceedings. 46: 183-189, 1987.

Ashwell, J.D.: Lymphocytes: properties and strategies. In The Liver: Biology and Pathobiology. Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A. (Eds.) Raven Press, New York. In press.

Ashwell, J.D., Longo, D.L., and Bridges, S.H.: T cell tumor elimination as a result of T cell receptor-mediated activation. Science. In press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09309-01 OAD

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Activation Requirements and Diversity of Gamma-Delta T Cells

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

PI:	A.M. Kruisbeek	Visiting Scientist	OAD, NCI
Others:	S. Marusic	Visiting Fellow	OAD, NCI
	M. A. Weston	Biologist	OAD, NCI

COOPERATING UNITS *(if any)*

Laboratory of Immunology, NIAID (R.H. Schwartz, R.N. Germain); Laboratory of Immunogenetics, NIAID (J. Coligan); Immunology Branch, NCI (J.A. Bluestone).

LAB/BRANCH

Office of the Associate Director

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Antigen-specific murine and human T cells express a polymorphic receptor, consisting of 2 disulfide linked chains, termed alpha and beta, noncovalently linked to a nonpolymorphic complex of proteins, termed T3. The T3 complex plays a vital role in the signal transduction process of T cells. Over the past year, a new type of T cell was discovered, expressing a T 3 complex linked to 2 new chains, termed gamma and delta. These cells express a double negative (CD4- CD8-) phenotype and are predominantly expressed in early T cell population. To achieve a better understanding of the activation requirements and expression of this new receptor at the clonal level, a panel of T cell hybridomas with gamma-delta receptors was established. Analysis of the activation pathways utilized by these cells demonstrated that they are responsive to the same signals (i.e., anti-T3, anti-Thy-1 and con A) as "conventional" alpha beta T cells. Furthermore, biochemical analysis of T cell hybridomas has demonstrated so far 2 different species of gamma chain and 3 different species of delta chain, thus revealing a diversity in both gamma and delta not expressed at the total population level (i.e., in double negative thymocyte populations from which the hybridomas are derived). Future studies include: (1) development of monoclonal antibodies specific for gamma and delta chains, such that in vivo and in vitro studies on the effect of such antibodies on gamma-delta cells and their role in T cell development can be explored; (2) development of antigen-specific gamma-delta T cells and hybridomas, such that possible ligands for this new receptor can be identified.

The significance of this projects lies in 2 areas: (1) understanding the role of gamma-delta cells in early T cell development, and (2) understanding why this new receptor is engaged in recognition of a wide variety of neoplasms.

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD NCI
Suzana Marusic	Visiting Fellow	OAD NCI
Margaret A. Weston	Biologist	OAD NCI

OBJECTIVES

1. A study expression of the T_3-gamma-delta-TCR-complex at the clonal level, to determine what signals activate gamma-delta cells, and to compare the spectrum of functional activities expressed by these cells with those of conventional alpha-beta cells;

2. To develop clones and hybridomas of antigen-specific T_3-gamma-delta cells;

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

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

MAJOR FINDINGS

Since the recent identification of the protein products of the gamma gene family on a new type of T cells in both mouse and man, much has been learned about their expression. The gamma chain is, on most gamma-expressing T cells, associated with a partner chain, termed delta. The gamma-delta heterodimer is associated with an invariant T_3-complex and appears predominantly, if not exclusively, on cells with a CD_4-CD8- phenotype both in the thymus and the periphery. Knowledge of the activation properties and potential diversity of T_3-gamma delta cells, are essential to our understanding of the physiologic role of this distinct T cell lineage. We have examined the T_3 components, activation properties, lymphokine production, and gamma and delta chain diversity of a panel of cloned T_3-gamma-delta T cell hybridomas. Our results demonstrate that, by both functional and biochemical criteria, activation through the T_3-gamma-delta complex isolated from these hybridomas is currently used to raise monoclonal antibodies. These antibodies will be used to explore the effect of in vivo and in vitro elimination of cells expressing the gamma-delta complex on normal T cell development.

PUBLICATIONS

Lew, A.M., Pardoll, D.M., Maloy, W.L., Fowlkes, B.J., Kruisbeek, A., Cheng, S., Germain, R.N., Bluestone, J.A., Schwartz, R.H., and Coligan, J.E.: Characterization of T cell receptor gamma chain expression in a subset of murine thymoctyes. Science 234: 1401-1405, 1986.

Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maloy, W.L., Coligan, J.E., and Schwartz, R.H.: Differentiation expression of two distinct T cell receptors during thymocyte development. Nature 326:79, 1986.

Pardoll, D.M., Lew, A.M., Maloy, W.L., Fowlkes, B.J., Kruisbeek, A., Bluestone, J.A., Schwartz, R.H., and Coligan, J.E.: Analysis of T cell receptor gamma chain expression in the thymus. Of Proteins and Peptides, in press.

Pardoll, D.M., Fowlkes, B.J., Kruisbeek, A., Coligan, J.E., and Schwartz, R.H.: The unfolding story of the T cell receptor gamma chain. Fed. Proc., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09310-01 OAD

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Early T cell development

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

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

COOPERATING UNITS *(if any)*

Laboratory of Immunology, NIAID (W.E. Paul);
Immunology Branch, NCI, (J.A. Bluestone)

LAB/BRANCH

Office of the Associate Director

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

CHECK APPROPRIATE BOX(ES)

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

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

The signals and growth factors required for the development of T cells are largely unknown. During early fetal thymus ontogeny, a variety of cell surface molecules are expressed which could potentially serve as transducers of activation signals. Two of such molecules (i.e., Thy-1, and the epsilon-component of the T3 complex) were explored and shown to function in early fetal thymocyte activation, as evidenced by both induction of proliferation and elaboration of lymphokines. We found that both IL-2 and BSF-1/IL-4 are produced upon activation of fetal thymocytes through Thy-1- and T3-activation, and are currently exploring, in an in vitro organ culture system, the effect of blocking IL-2 or IL-4 usage on T cell development. 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. It was demonstrated furthermore, that in the early fetal thymus, all T3-expressing cells are of the gamma-delta phenotype.

The significance of this project lies in: (1) understanding the factors that control the development of T cells, and (2) application of this knowledge to studies on immune reconstitution.

PROJECT DESCRIPTION

PERSONNEL

Ada M. Kruisbeek	Visiting Scientist	OAD NCI
Lucio Tentori	Visiting Fellow	OAD NCI
Margaret A. Weston	Biologist	OAD NCI

OBJECTIVES

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

2. To explore in vivo and in vitro models 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 T3-complex as transducers of activation signals. In addition, we demonstrated that fetal thymocytes produce both IL-2 and IL-4 upon activation. As these studies were performed at a timepoint before conventional alpha-beta receptor bearing cells are present, the results suggest a role for early gamma-delta receptor bearing cells in development, i.e., production of lymphokines. Current studies focus on: (1) which other cell surface molecules are involved in the delivery of successful activation signals to early T cells; and (2) which role the gamma-delta cells play in the development of alpha-beta cells.

PUBLICATIONS

None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09311-01 OAD

PERIOD COVERED

October 1, 1986 to September 30, 1987

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 Staff Fellow	OAD, NCI
Others:	M. Mercep	Visiting Fellow	OAD, NCI
	J. Sussman	Howard Hughes Medical Scholar	

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology and Metabolism, NICHHD (J. Bonifacino and R. Klausner), Laboratory of Immunology, NIAID (T. Saito and R. Germain)

LAB/BRANCH

Office of the Associate Director

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

To analyze the contribution of individual T cell antigen receptor (CD3/Ti) components, CD3/Ti variants were generated. MA 5.8, a subclone of the pigeon cytochrome c-specific T cell hybridoma 2B4.11, was produced by mutagenesis with EMS followed by selection in the presence of antigen and antigen-presenting cells. MA 5.8 failed to express any cell surface or internal CD3-zeta chain. Pulse-chase studies found that MA 5.8 did not synthesize detectable CD3-zeta. Furthermore, the half-life of other CD3 subunits, notably delta and epsilon, was considerably shortened. Survival was dramatically increased by the addition of ammonium chloride, implying that the degradation of these subunits was enhanced in the absence of CD3-zeta. Fluorescent antibody staining found that MA 5.8 expressed approximately 20-fold less of the other CD3/Ti components on its surface. In spite of this, MA 5.8 did respond to plastic-adherent anti-CD3 or anti-Ti monoclonal antibodies (mAb) by producing IL-2. However, when compared to 2B4.11 or 2B4.3.12 (another 2B4 subclone that expressed 20-fold less cell surface CD3/Ti but that synthesied CD3-zeta), MA 5.8 responded poorly. In fact, MA 5.8 made barely detectable IL-2 when stimulated with antigen, and no IL-2 when stimulated with a mitogenic anti-Thy-1 mAb. Another 2B4.11 variant, 21.2.2, spontaneously lost 3 or 4 Ti chains, and consequently failed to express CD3/Ti. When 2 of these chains were transfected back into this cell (yielding the cell T 1.2), CD3/Ti expression was regained. 2B4.11 and T 1.2, but not 21.2.2, produced IL-2 in response to stimulation with an anti-Thy-1 or an anti-Ly-6 mAb. Despite this, neither 21.2.2 nor T alpha beta 1.2 manifested increases in intracellular Ca2+ or phosphatidylinositol metabolites. These results suggest that products of phosphatidylinositol metabolism are either not involved in IL-2 production, or one effective at levels below the limits of detection. Attempts will be made to generate other CD3/Ti variants that have modified or missing CD3/Ti components, and to analyze the expression and function of the remaining subunits.

PROJECT DESCRIPTION

PERSONNEL

Jonathan D. Ashwell	Senior Staff Fellow	OAD, NCI
Mladen Mercep	Visiting Fellow	OAD, NCI
Jeffrey Sussman	Howard Hughes Medical Scholar	

OBJECTIVES

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

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

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. Generation of T cell Antigen-receptor Expression Variants To Study The Role The Individual Components

The T cell antigen-specific receptor has two major subunits: the antigen-binding heterodimer (Ti) composed of the alpha and beta chains, and the CD3 complex, composed of the gamma, delta, epsilon, zeta, and p21 chains. At present, no variants of this CD3/Ti complex have been available to allow the dissection of the functions of the individual chains. In order to generate such cells, the T cell hybridoma 2B4.11 was co-cultured with the mutagen ethyl methane sulfonate (EMS) for 18 hours and then plated into tissue culture wells at a density of 10^4 cells/well in the presence of antigen (pigeon cytochrome c) and syngeneic antigen-presenting cells. As described in detail in the project Antigen Presentation and T Cell Activation, stimulation of T cell hybridomas with antigen causes an irreversible block at the G_1/S interface, and provides a useful means of selecting those T cells that had either had CD3/Ti components lost or modified. Although the great majority of the cells died, about 60% of the wells contained cells that expanded with time. The cells in one such well were cloned by limiting dilution, and one subclone, MA 5.8, was chosen for further study.

Analysis by fluoresceinated monoclonal antibody staining and flow cytometry found that MA 5.8 bore approximately 20-fold less Ti-alpha and CD3-epsilon on its surface than did the parent 2B4.11 cell. Northern blot analysis demonstrated that MA 5.8 bore normal levels of mRNA for the two Ti chains, alpha and beta. In order to determine if the reason for the decreased expression of CD3/Ti could be a defect in one or more of the CD3 components, cell surface or total membranes were radiolabelled with ^{125}I. Immunoprecipitation with antibodies directed against each of the CD3 components followed by SDS-PAGE detected all of the CD3/Ti subunits with the exception of CD3-zeta. To determine if this were due to decreased production or enhanced degradation, biosynthetic labeling and pulse-chase studies were done. It was demonstrated

that MA 5.8 did not synthesis detectable amounts of CD3-zeta, although the other subunits were manufactured at normal levels. Interestingly, pulse-chase followed by immunoblotting established that the survival of at least two other CD3 components, delta and epsilon, also depended upon the presence of zeta. Whereas these two components are present at about 5% to 8% of the synthesized amount for prolonged periods (up to 10 hours), in MA 5.8 less than 1% of CD3-delta and -epsilon survived for 10 hours. That this was probably the result of enhanced degradation was shown by the fact that ammonium chloride, which raises lysosomal pH, prevented the abnormally rapid loss of these CD3 subunits. These data represent the first direct evidence that an intact CD3 is necessary for normal Ti expression, and suggest that CD3-zeta plays a role in the assembly of the complex.

The functional properties of this CD3-zeta negative T cell were also assessed. To control for changes that might be due to the fact that MA 5.8 expressed only about 5% as much CD3/Ti on its surface as did 2B4.11, another variant of 2B4 was obtained. 2B24.3.12 was derived by repetitive limiting dilution cloning and screening for low CD3/Ti expressors. 2B4.3.12 bears as little CD3/Ti on its surface as does MA 5.8, but has normal intracellular levels of CD3-zeta. All of the other CD3 components are also present, although CD3-delta appears to be substantially decreased. Whether this is the reason for the lack of normal CD3/Ti expressors is under investigation. Stimulation with either the mAb 2C11 (anti-CD3-epsilon) or A2B4-2 (anti-Ti-alpha) bound to plastic caused 2B4.11, 2B4.3.12, and MA 5.8 to produce IL-2. However, comparison of the maximal amounts of IL-2 generated revealed that, 2B4.3.12 made ~35% and MA 5.8 made ~10% as much IL-2 as 2B4.11. Stimulation with 2C11 cross-linked by the Fc receptor bearing B cell hybridoma LS found an even greater dichotomy, with 2B4.3.12 making ~15% as much IL-2, and MA 5.8 ~2%. Surprisingly, stimulation with antigen revealed that whereas 2B4.3.12 responded relatively well (producing ~5% as much IL-2), MA 5.8 barely responded. In fact, when the data were normalized to compare does-response curves, 2B4.3.12 was perhaps 3 to 10-fold less sensitive to antigen than 2B4.11, whereas MA 5.8 was at least 100,000-fold less sensitive. Antigenic stimulation was not unique in this regard. When stimulated with the mitogenic anti-Thy-1 mAb G7, 2B4.3.12 produced about 15% as much IL-2 as did 2B4.11, whereas MA 5.8 made no detectable IL-2, even at 1,000-fold increased levels of the mAb. Together, these data demonstrate that CD3-zeta is not required to transduce the signals that culminate in IL-2 production. However, in the absence of CD3-zeta the function of CD3/Ti is impaired. Furthermore, it appears that the degree to which it is impaired depends upon the nature of the signal, with highly efficient CD3/Ti cross-linking (by mAb-coated plastic wells) being the least affected, and antigen or anti-Thy-1 mAb stimulation affected most profoundly.

It should be noted that without any genetic information available for CD3-zeta, it is not possible to completely and unequivocally characterize MA 5.8's lesion. Only when CD3-zeta has been cloned and transfected back into MA 5.8 will it be possible to unequivocally state that it was the failure to express CD3-zeta that led to the defects described here. Future efforts will be directed toward this end. Furthermore, 2B4.3.12 will be analyzed further to characterize its defect, and, if truly deficient in CD3-delta, will be subcloned in an attempt to produce a completely CD3-delta negative T cell. Since CD3-delta has been cloned, the sort of transection studies mentioned above should be feasible.

II. Transmembrane Signalling Events in T Cells

In the course cloning 2B4.11, a completely Ti-negative subclone was derived (termed 21.2.2). Northern blot analysis found that 21.2.2 had lost 3 of the 4 Ti chains: 2B4 alpha, 2B4 beta, and BW5147 beta, retaining only BW5147 alpha chain mRNA. As has been shown with other T cells, the failure to express cell surface Ti also precluded the cell surface expression of CD3. Attempts to stimulate 21.2.2 to produce IL-2 using G7 or a mitogenic anti-Ly-6 mAb, D7, were unsuccessful, although 21.2.2 did produce IL-2 when stimulated with PMA, whose activity is thought to bypass cell surface receptors. To determine if it was only the loss of CD3/Ti expression that affected the ability to produce IL-2, 21.2.2 was sequentially transfected with the 2B4 alpha and 2B4 beta chains in drug resistance vectors, restoring expression of both Ti and CD3. This transfectant, called T 1.2, once again produced IL-2 in response to G7 and D7, providing that it was CD3/Ti expression, and not that of some other molecule, which was essential for this response to mAb perturbation of "activation antigens."

In addition to IL-2 secretion, we analyzed the ability of these cells to generate early activation signals. Antigenic stimulation of T cells results in a variety of early events, including increases in intracellular free Ca^{2+} and phosphatidlyinositol hydrolysis to inositol phosphates. 2C11, G7, and D7 all caused these same increases in the 2B4.11 parent cell. 21.2.2, however, failed to generate either Ca^{2+} or inositol phosphate (IP1, IP2, or IP3) increases. To our surprise, T 1.2 also failed to manifest either of these responses despite the fact that it produced large quantities of IL-2. We indirectly examined another result of phosphatidlyinositol metabolism, that is, the generation of diacylglycerol and the activation of protein kinase C. The CD3-gamma subunit is phosphorylated in response to G7 stimulation, an effect that is thought to be mediated by protein kinase C. Labeling with ^{32}P, stimulation with G7, and specific immunoprecipitation and analysis by NEPHGE (non-equilibrium pH gel electrophoresis), revealed that CD3-gamma was phosphorylated to a much lesser extent (preliminary estimates ~25%) than in 2B4.11. Together, these data suggest that phosphatidylinositol metabolism is probably not involved in IL-2 production by 2B4.11. However, if it is involved, the levels that are required to generate the entire dose-response curve are below that detectable by the standard techniques. This appears to be true for intracellular Ca^{2+} increases as well.

SIGNIFICANCE

Little is currently known about the "activation events" that occur in T lymphocytes after the antigen-specific T cell receptor is engaged. T cell receptor mutants and variants provide a powerful tool with which to study structural, (i.e., how the T cell antigen receptor is assembled and expressed), and functional, (i.e., which components are necessary for transducing signals), requirements. By analyzing their defects, and ultimately by correcting them with the transection of the missing or mutated genes, one should be able to generate a comprehensive understanding of both the roles played by the individual components, and the manner in which the assembled complex functions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09289-02 LMI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Role of Lymphokines and Cytokines in B Cell Immunity and Hematopoiesis

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

PI:	J. J. Oppenheim	Chief	LMI, NCI
Others:	K. Matsushima	Visiting Associate	LMI, NCI
	M. Kawano	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

Armed Forces Radiobiology Research Institute, Bethesda, MD (R. Neta);
PRI, NCI-FCRF (R. Apte, I. Kedar).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

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INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

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

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

This laboratory has been focusing its efforts on the role of lymphokines and cytokines in hematopoiesis and immune system development. Specifically, we have focused on 1) the biochemical characterization of a lymphokine produced by T cells which stimulates B cells to proliferate and 2) the role of specific lymphokines (i.e. IL 1, CSFs) and cytokines (i.e. TNF) in the protection of bone marrow from lethal doses of irradiation. A high molecular weight lymphokine produced by lectin stimulated T lymphocytes that stimulates B cell proliferation has been characterized and highly purified. This BCGF was free of interleukin 1 and 2 (IL 1 and 2) activities, but could induce B cells to proliferate and to express receptors for IL 2. This lymphokine could also support the growth of BCL 1 cells, a unique property of BCGF-II and could augment the proliferation of a large granular lymphocyte line (YT), several EBV +B cell lines and murine thymocytes.

In collaboration with scientists at AFRRI we have demonstrated that in vivo administration of recombinant IL 1β or IL 1α, 20 hrs prior to lethal doses of irradiation, is protective. Mice pretreated with IL 1 do show a partial recovery in the number of nucleated bone marrow cells and show evidence of increased erythropoiesis by colony forming assays (CFU-E), whereas there is no such recovery in untreated control mice. Bone marrow from IL 1 treated mice demonstrated increased proliferation when subquently cultured for 3 days with GM-CSF. In contrast, administration of several recombinant lymphokines that are induced by IL 1, namely GM-CSF, IL 2 and immune interferon, 20 hrs and 3 hrs before irradiation had no protective or proliferative effect. Prostaglandins are also not effective. Although neither G-CSF nor CM-CSF were effective by themselves, these CSF's in combination with suboptimal doses of IL 1 had synergistic radioprotective effects. Finally TNF, although not as potent as IL 1, also was observed to be radioprotective by itself. IL 1 and TNF together yielded additive effects, suggesting that their mechanism of radioprotection differs.

PROJECT DESCRIPTION

PERSONNEL

Joost J. Oppenheim	Chief, LMI	OC	LMI	NCI
Kouji Matsushima	Visiting Associate	OC	LMI	NCI
Michio Kawano	Visiting Fellow	OC	LMI	NCI

MAJOR FINDINGS

I. Effect of BCGF II on Human B Cells

We have investigated and characterized several lymphokines that influences B cell proliferation.

A bioassay was developed using human small B cells adherent to anti-human IgM (anti-μ)-coated microtiter dish wells. These B cells were stimulated to proliferate by culture supernatants of concanavalin A (Con A)-activated human peripheral blood lymphocytes (Con A Sup) even in the presence of high concentrations of anti-μ coated on assay wells. Human B cell growth factor (BCGF) activities were partially purified from Con A Sup. Preparative chromatography (Sephacryl S-200 and isoelectrofocusing) yielded a major peak of BCGF activity for B cells adherent to anti-μ-coated wells with a m.w. of 50,000 (50 Kd) and a pI 7.6. The 50 Kd BCGF was further purified by sequential chromatography using DEAE-Sephacel, CM-Sepharose, Sephacryl S-200, CM-high performance liquid chromatography (HPLC) and hydroxyapatite (HA)-HPLC. The HA-HPLC purified 50 Kd BCGF was free of IL 1, IL 2, and interferon activities, but could support growth of BCL1 cells, similar to BCGF-II. Neither IL 1 nor interferon γ had any growth stimulating effect in our B cell proliferation assay with or without BCGF in Iscove's synthetic assay medium.

BCGF-induced proliferation of B cells adherent to anti-μ-coated wells could be markedly augmented by the simultaneous or sequential addition of recombinant human IL 2 (rIL 2). When cultured for 3 days with 50 Kd BCGF, about 40% of B cells adherent to anti-μ-coated wells expressed Tac antigen, and anti-Tac monoclonal antibody inhibited rIL 2-enhancement of proliferation of 50 Kd BCGF-preactivated B cells. We have therefore identified a major 50 Kd BCGF activity with Tac antigen-inducing activity that also has a synergistic effect with IL 2 on normal B cell proliferation.

II. Effects of BCGF II on Other Lymphoid Cells

Many cytokines have been documented to have a multiplicity of biological effects by acting on a variety of cells. In order to determine whether human BCGF II acts on any cells in addition to normal B cells, the effect of human BCGF II on murine thymocytes, human peripheral blood T cells, a human natural killer-like cell line, YT, and Epstein-Barr virus (EBV)-transformed B cell lines was further examined. BCGF II augmented incorporation of 3H-thymidine by murine thymocytes in combination with suboptimal doses (0.5 μg/ml) of Con A but not at lower doses (0.1 μg/ml) of Con A, a concentration usually used for IL 1 assays. BCGF II could not induce either proliferation or Tac

antigen (Ag) expression on normal peripheral blood T cells stimulated with OKT3 antibody. However, both proliferation and Tac Ag expression on YT cells were also augmented by BCGF II. BCGF II induced both high and low affinity IL 2 receptor (IL 2R) on YT cells as determined by 125I-IL 2 binding assay. Two of 7 EBV-transformed B cell lines tested (ORSON and AUM cells) exhibited augmentation of proliferation and cell surface Tac Ag expression in response to BCGF II. BCGF II in the presence of low doses (0.1 μg/ml) of phorbol myristate acetate (PMA) also induced Tac Ag mRNA (3.5 and 1.5 kb) in these B cell lines. The IL 2R induced on these B cell lines, however, consisted mostly of low affinity receptors. Both Tac Ag and its mRNA in these B cell lines were induced by PMA but not forskolin, suggesting that this induction may involve protein kinase C. The present study shows that human BCGF II can stimulate YT cells, murine thymocytes and some EBV-transformed B cell lines but not peripheral blood T cells. Consequently, BCGF II can induce the growth and differentiation of a number of cell types in addition to normal B cells.

III. Studies of Radioprotection by IL 1

In collaboration with scientists at AFRRI, we have identified several cytokines with radioprotective effects and studied the mechanism by which they may be protecting hematopoiesis. Murine IL 1α and human IL 1α and IL 1β protect mice from lethal effects of radiation-induced hematopoietic syndrome. A single 100 ng dose of h IL 1α conferred protection, provided it was administered to C57BL/6 and DBA/1 mice 20 hrs before irradiation with an LD 100/30 dose, with a DRF of 1.25 and 1.2, respectively. Studies of possible mechanisms of radioprotection by IL 1 showed that radioprotection of LD 100/30 irradiated mice could not be induced with IL 2, IFNγ or GM-CSF. Radioprotection with IL 1 did not depend on the release of prostaglandins, since indomethacin did not diminish survival of IL 1 treated mice. Unlike C57BL/6 and DBA/1 mice, C3H/HeJ mice were not protected from lethal irradiation by IL 1. Nevertheless both high responder C57BL/6 and low responder C3H/HeJ strains, treated with IL 1, develop similarly enhanced levels of acute phase proteins: metallothionein and ceruloplasmin, with known radioprotective abilities. Therefore, the observed differences in radioprotection with IL 1 in murine strains probably can not be attributed to differences in levels of these scavenging metalloproteins.

IV. Hematopoietic Effects of IL 1

The recovery of total nucleated bone marrow cells and of hematopoietic progenitor cells was enhanced in IL 1 treated, as compared to untreated, irradiated mice. This suggested that IL 1 administration may effect the cells in the bone marrow of normal mice. Intraperitoneal administration of recombinant IL 1 resulted in bone marrow cell enlargement and increased cycling of these enlarged cells. In addition, the capacity of bone marrow cells from IL 1 treated mice to proliferate in response to GM-CSF in cell suspension cultures was enhanced. The above effects were not genetically restricted as C57B1/6, B6D2F1, C3H/HeN and C3H/HeJ mice showed similar responses. A comparative study showed that 100 ng of IL 1 was much more effective in stimulating bone marrow cells by the above criteria than 5 ug GM-CSF. Since IL 1, unlike CSF, can not be demonstrated to have a direct in vitro stimulatory effect on bone marrow cells, the aforementioned in vivo effects of IL 1 are presumably mediated by other hematopoietic growth factors. We have previously shown that IL 1 induces the appearance of

high titers of CSF in the serum. Consequently hematopoietic growth factors that are generated at local sites following IL 1 administration may mediate the observed cell cycling effect.

V. Interactions Between IL 1, CSF and TNF

The hypothesis that IL 1 may be restoring hematopoiesis by generating CSF was tested by evaluating the radioprotective effects of administering these lymphokines in combination. Doses of GM-CSF or G-CSF, which were inactive by themselves, when administered intraperitoneally together with suboptimal doses (33 ng of IL 1α or β) yielded synergistic radioprotective effects. This, together with the observation that IL 1 administration leads to the appearance of detectable circulating levels of CSF in mice, suggests the possibility that these cytokines act in an intelligent manner in vivo to promote hematopoesis. Additional studies have revealed that tumor necrosis factor (TNF), which shares many of the activities of IL 1, is also radioprotective. However, higher doses, 5-7.5 ug IP, of human recombinant TNF than IL 1 are needed to obtain a radioprotective effect. Combined administration of IL 1 and TNF yields additive radioprotective effects suggesting that the mechanism of action of these two cytokines differs.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1986 to September 30, 1987

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

Oncogenes in Cellular Transformation and Activation.

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

PI:	L. Varesio	Visiting Scientist	LMI, NCI
Others:	M. Clayton	Microbiologist	LMI, NCI
	E. Blasi	Visiting Associate	LMI, NCI
	G. Gusella	Visiting Fellow	LMI, NCI
	L. Gandino	Visiting Fellow	LMI, NCI
	A. Carbone	Guest Researcher	LMI, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (S. Giardina); Experimental Therapeutics Section, LEI, NCI (R. Wiltrout); LBI, NCI (M. Barbacid); Food & Drug Administration, (E. Bonvini); USUHS (J. Mond); PRI, NCI-FCRF (D. Radzioch, A. Leyko).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Immunobiology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This laboratory has been focusing its efforts on the biochemical and molecular events which occur upon macrophage activation. Studies were performed on macrophage cell lines immortalized by recombinant retroviruses expressing the oncogenes v-myc and v-raf. It has been demonstrated that macrophage lines retain the ability to express, constitutively or upon stimulation, the immunoregulatory and effector functions of normal peritoneal macrophages. The expression of the proto-oncogene c-fos, was also induced by activators of protein kinase C (PK-C) with modalities similar to normal macrophages. These results indicated that expression of viral oncogenes does not affect macrophage function and thus establishes these cell lines as suitable for investigation of the molecular basis of macrophage function.

We have previously demonstrated a clear association between accumulation of ribosomal RNA precursors and expression of cytotoxic activity by macrophages. We have now found that this molecular event is a common endpoint to the pathways of macrophage activation induced by interferon alpha, beta, or gamma. Additional experiments have revealed, however, that IFN β and γ differ in their effects upon c-fos activation indicating that the initial steps involved in macrophage activation by these agents may differ.

Based on our studies on the expression of the c-fos proto-oncogenes in macrophages, we have designed experiments aimed at the understanding the relationship between transforming oncogenes and the c-fos proto-oncogene. These studies have revealed that cells transformed by Ha-ras, but not Ki or N-ras, lose the ability to express c-fos in response to PKC activators. These data indicated that the mechanism of cellular transformation by H-ras may involve deregulation of c-fos functions as a consequence of altered cellular responses to external stimuli.

PROJECT DESCRIPTION

PERSONNEL

Luigi Varesio	Visiting Scientist	IS	LMI	NCI
Michael Clayton	Microbiologist	IS	LMI	NCI
Elisabetta Blasi	Visiting Associate	IS	LMI	NCI
Gabriele Gusella	Visiting Fellow	IS	LMI	NCI
Lucia Gandino	Visiting Fellow	IS	LMI	NCI
Arnaldo Carbone	Guest Researcher	IS	LMI	NCI

OBJECTIVES

The objectives of the research have been: 1) to develop a functional macrophage cell line suitable for gene transfer experiments; 2) to investigate the mechanisms of macrophage activation by interferon; and 3) to determine the interrelationships among oncogenes in the immortalization and transformation of various cell types. The major findings can be summarized as follows:

MAJOR FINDINGS

I. Immortalization of Macrophages with Recombinant Retrovirus Carrying myc and raf Oncogenes

We have previously reported that a recombinant murine retrovirus expressing v-myc and v-raf oncogenes (J2) can immortalize bone marrow cells (BM) of C3H/HeJ mice and give rise to macrophage cell lines. Extensive investigation has focused on determining whether this experimental system could be used as a general method to consistently generate macrophage lines or whether other cell types could be immortalized. Viruses containing v-raf or v-myc alone failed to induce BM proliferation in 24 out of 27 experiments performed as only the virus containing both v-raf and v-myc oncogenes induced BM proliferation. Thus, co-expression of the two oncogenes was needed to provide the mitogenic signal(s) for BM proliferation. Exogeneous growth factors (GF) were not required to sustain the mitogenic effect of J2 virus. The kinetics of growth of the J2 virus-infected cells (J2 cells) were characteristically biphasic. The initial burst of proliferation was always followed by a quiescent phase culminating in cell death, which could not be reversed by addition of exogenous GF. In contrast, active proliferation of the quiescent monolayers could be restored by addition of dextran-based beads to the cultures, showing that the growth arrest of J2 cells was a reversible process. J2 cells actively growing in the presence of CT-beads could be expanded and cloned, and subsequently grew continuously independent of the CT-beads. Eighteen clones obtained from different infections were all macrophages by morphological criteria and all of them expressed the same membrane phenotype compatible with macrophage, demonstrating that J2 virus infection leads to immoralization of the same BM-derived monocytic subpopulation. When injected in vivo, J2 cells produced histiocytic tumors in nude mice, but did not grow in immunocompetent syngeneic mice. The cells induced to proliferate in vitro in response to J2 virus infection appeared to be limited to the BM compartment, since spleen cells, thymocytes, peritoneal macrophage and liver large granular lymphocytes (LGL) did not grow in vitro in response to J2 the virus. We concluded that the immortalization of BM

cells by J2 virus infection represents a novel reproducible experimental system to deliberately generate macrophage lines, which proliferate in response to viral oncogenes and do not require exogenous GF to initiate or to sustain their continuous proliferation.

One of the practical uses of such macrophage cell lines was the establishment of an in vitro system to analyze the molecular basis for the expression of functional activities by macrophages. Therefore, we studied the ability of clones of macrophage cell lines for their ability to express macrophage functions constitutively or upon activation by BRMs. All of the clones obtained had macrophage-like phenotypes, and one such clone, GG2EE, has been compared to normal macrophage to ascertain the effects of immortalization on the expression of the biological functions of the lines. GG2EE cells expressed cytotoxic activity against various tumor target cells in response to stimulation with interferon γ (IFN γ) and heat-killed Listeria monocytogenes. GG2EE cells did not constitutively express I-A or I-E antigens; nevertheless, I-region coded antigens could be induced by IFN γ treatment. GG2EE cells produced interleukin 1 upon stimulation with a T cell-derived lymphokine; they were weakly phagocytic, yet became highly phagocytic following IFN γ treatment. Since c-fos mRNA is augmented in peritoneal exudate macrophage by protein kinase C activators but not by IFN γ, we evaluated the effects of calcium ionophore (Ca++I), phorbol myristate acetate (PMA), L-α-1-oleoyl-2-acetoyl-sn-3 glycerol (OAG) and IFN γ on the levels of c-fos mRNA in GG2EE cells. We found that Ca++I, PMA and OAG stimulation enhanced the expression of c-fos mRNA, but IFN γ treatment did not. The kinetics of c-fos induction in GG2EE cells were also comparable to those observed in peritoneal exudate macrophage. Overall, the GG2EE cell line has the same biological properties as normal tissue macrophage. Because it is capable of both constitutive and inducible macrophage-like functions, this cell line provides a valuable tool for studying the molecular mechanisms controlling macrophage activation.

Because of the differential expression of c-fos and c-myc during proliferation and differentiation of myelomonocytic cells, we decided to study the biological effects of a retrovirus expressing the combination of oncogenes v-raf and v-fos. We expect that with this recombinant retrovirus we may be able to immortalize lymphoid cells or macrophages at a stage of differentiation distinct from the cell lines generated with the J2 virus. To obtain a v-raf/v-fos recombinant retrovirus we substituted the v-myc of the J2 virus with the v-fos oncogene derived from the FBJ-2 virus. Most of the v-myc oncogene was deleted by eliminating a 3000 bp DNA fragment from the J2 virus. The deletion of this portion of the J2 genome produced inactivation of the splicing acceptor site (SAS) between v-raf and v-myc. Therefore, in order to assure the correct processing of the viral RNA, the introduction of a new SAS was necessary. For this purpose a 441 bp region of the Moloney leukemia virus containing the SAS of the env gene, was subcloned in the pGEM4 vector and the v-fos gene was ligated downstream from this splicing consensus sequence. Finally, the whole SAV/v-fos fragment was inserted in the ClaI site of the J2 virus from which the v-myc was excised. This v-raf/v-fos construct is being used to transform the NIH 3T3/2 and the NIH 3T3/Am22b cell lines containing encapsidation-defective murine and amphotropic retroviral sequences respectively. Such defective viral sequences provide the same complementing functions of a helper virus without having the ability to be encapsidated themselves because of a mutation affecting their

replication sites. The NIH 3T3/2 cell line will provide a murine specific virus while the NIH 3T3/Am22b cells will give an amphotropic virus, thus capable of infecting cells from different species. The transforming ability of the defective v-raf/v-fos virus will then be tested on murine and human BM cells.

II. Expression of c-fos Proto-oncogene and its Possible Biological Relevance in Myelomonocytic Cells

Central to the studies on proto-oncogenes is the delineation of their role in cell biology and expression of specialized functions. The pleiotropic effects of c-fos could be a lineage-specific response, proliferative in fibroblasts and differentiative in other cell types and different biochemical pathways may lead to augmentation of c-fos mRNA. We analyzed the modulation of c-fos mRNA in fresh peritoneal exudate macrophages, which are non-cycling terminally differentiated macrophages. The levels of c-fos mRNA are drastically increased by stimulation with 12-O-tetadecanoyl phorbol-13-acetate (PMA), L-α-oleoyl-2-acetoyl-sn-3-glycerol (OAG) or calcium ionophore. The demonstration that OAG, PMA and calcium ionophore are inducers of c-fos mRNA raised the question of whether augmentation of c-fos mRNA was a common event associated with macrophage stimulation. To address this point we examined the effect of IFN β and IFN γ on c-fos expression, since both cytokines are potent activators of macrophage. No significant changes in the levels of c-fos mRNA were observed in response to IFN, indicating that augmentation of c-fos mRNA was not a general feature of stimulation but a selective response to PMA, OAG and calcium ionophore. Different biological responses followed stimulation of macrophage with the various agents: chemotactic response was elicited by PMA and OAG, superoxide production by PMA and cytotoxic activity by IFN β, IFN γ, and calcium ionophore. These results showed that the stimuli used were all biologically active since they were able to trigger various functions. However, we did not find a correlation between the ability of a stimulus to induce a specific function and to augment c-fos mRNA expression. Overall, we demonstrated that c-fos mRNA is susceptible to augmentation in primary cultures of terminally differentiated macrophage. Since the agents tested are direct or indirect activators of PK-C and our data suggest that in macrophage c-fos is controlled by PK-C activation. However, augmentation of c-fos oncogene expression does not seem to be a signal sufficient to induce the manifestation of macrophage functions since no such correlation was found.

It was possible that the constitutive baseline levels of c-fos mRNA were important for macrophage activation. Constitutive expression of c-fos proto-oncogene was inhibited by H7, a specific inhibitor of PK-C, suggesting that constitutive levels of c-fos were maintained by PK-C activity. To assess whether the constitutive levels of c-fos oncogene were related to the activation of macrophage, we tested the ability of IFN to activate H7-treated macrophage. We found that H7, under conditions in which it inhibits c-fos expression, blocked the activation of macrophage induced by IFN β but not IFN γ. These results showed that the activation of macrophage by IFN γ differ from the activation by IFN β in the requirement for PK-C activity and/or constitute c-fos expression. Constitutive, c-fos expression may be a prerequisite for the ability of macrophage to be activated by IFN β. Experiments are in progress to determine whether other biological function of macrophage are affected under the condition of inhibition of c-fos expression by drugs or by anti-sense c-fos RNA.

III. Molecular Events in Macrophage Activation

Macrophage activation by IFNs leads to the expression of cytotoxic activity against tumor cells. The process of macrophage activation is associated with augmented enzymatic and secretory activities, expression of membrane antigens as well as alterations in metabolic pathways. Our studies on the molecular basis for activation of macrophages by IFNs to a tumoricidal stage have revealed that the RNA metabolism of macrophage is altered during the activation. Previous studies in our laboratory indicated that a constant feature of macrophages activated to cytotoxicity is down-regulation of RNA synthesis. Following this observation we investigated whether the synthesis of any particular RNA species is selectively inhibited. Ribosomal RNA rRNA analysis indicated down-regulation of 28S rRNA synthesis and normal levels of 18S rRNA in macrophages activated to a cytotoxic stage by IFN α. β and γ. Since 28S rRNA and 18S rRNA species are derived from high molecular weight rRNA precursors we estimated the levels of rRNA precursors in activated macrophages. The levels of rRNA precursors were measured by Northern blot analysis utilizing subclones of murine rDNA transcribed spacers as probes which recognize rRNA precursor molecules. We have consistently observed a high accumulation of 45S, 36S rRNA precursors and a lower but significant increase in 41S rRNA in murine peritoneal macrophage activated by IFN α, β and γ. In conclusion, our data show that the activation of macrophage to a cytotoxic stage is associated with the changes in rRNA metabolism and indicate that, in macrophage, the complex enzymatic interactions that coordinates the expression of the ribosomal genes is an intracellular target of IFN action.

IV. "Nurse Activity:" A New Macrophage Function

During the studies on the immortalization of bone marrow (BM) cells, experiments were performed in which macrophage and BM cells were cocultured. Studies on these mixed populations revealed a new macrophage activity exerted on the survival of BM cells. The survival of fresh BM cells in vitro was evaluated by measuring 111 indium (111 In) release from prelabeled BM cells. Upon 24 hr of culture in conventional medium, more than 50% of BM cells died. In order to investigate whether BM cell death could be reduced by coculture with other cell types, 111 In-labeled BM cells were incubated for 24 hr with peritoneal macrophage, thymocytes (THY), or polymorphonuclear cells (PMN) and then assayed for their survival. We found that coculture of BM cells with macrophage dramatically increased BM survival, whereas THY or PMN consistently failed to enhance BM survival. The ability to promote BM cell survival, designated "nurse" activity, represented a novel function of macrophage and was further characterized. The stage of activation of macrophage did not influence their nurse activity, since macrophage elicited in vivo by proteose-peptone, thioglycollate, or Bacillus Calmette-Guerin, as well as resident macrophage unstimulated or activated in vitro with lipopolysaccharide, equally sustained survival of BM cells. BM-derived macrophage (adherent cells from BM cultures maintained in 20% L-cell-conditioned medium for 14 days) were equally effective in exerting nurse activity. We propose that the nurse effect of macrophage on BM is a primitive function that may play an important role in the development of the hemopoietic system.

V. Effects of Transforming ras Oncogenes on the Induction of c-fos by the Phorbol Ester TPA

These studies were undertaken to explore the possible relationship between transforming oncogenes of the ras family and expression of c-fos proto-oncogene that may control the proliferation of fibroblasts. We studied the expression of the c-fos proto-oncogene since c-fos may control fibroblast proliferation in normal or transformed NIH-3T3. Treatment of exponentially growing 3T3 with 30 nM PMA causes transient increases in c-fos mRNA which peak at 15-30 min after treatment. However, no augmentation of c-fos mRNA could be induced by PMA treatment in H-rasH transformed 3T3 (115 and 44-9, two cell lines transformed by over-expression or a mutation of H-rasH respectively). The level of c-fos protein present in the cells before and after induction was measured by flow cytometry on permeabilized cells using mouse MoAbs against a 24 AA synthetic peptide (AA 128-151) of the fos protein. Low but detectable c-fos nuclear protein expression was present in unstimulated 3T3 fibroblasts. In contrast, 115 cells expressed high levels of endogenous c-fos. Following PMA treatment the levels of c-fos protein were increased in 3T3 but unaffected in 115 cells. The number of PMA binding sites was similar in 3T3 and 115 cells (7-8 x 10 5 sites/cell). However, the affinity of PMA binding to 115 cells was lower than for 3T3 fibroblasts (Kd = 96 and 50 nM, respectively). In conclusion, since the cell lines differ only with respect to the presence or absence of rasH, these results show that the transforming H-rasH in 3T3 fibroblasts induces constitutively high levels of c-fos expression and inability of PMA to further augment c-fos levels. We speculate that the change in affinity of the protein kinase C (PMA receptor) in H-rasH-transformed 3T3 may be related to the high constitutive levels of c-fos and/or the lack of c-fos augmentation in response to PMA. De-regulation of c-fos may contribute to the H-rasH induced transformation.

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

Role of Interleukin 1 in Immunity and Tumor Cell Growth

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

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	Y. Kobayashi	Guest Researcher	LMI, NCI
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COOPERATING UNITS *(if any)*

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Laboratory of Molecular Immunoregulation

SECTION

Immunobiology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This laboratory has focused its efforts on the biochemical characterization of the effects of IL 1 on target cells. Specifically, we have been analyzing the control of IL 1 receptor expression and the intracellular events which occur after IL 1 treatment of target cells. We have previously reported the existence of interleukin 1 (IL 1) receptors on EBV transformed human B lymphocytes, and the identity of the receptor for IL 1α and IL 1ß. We also determined that IL 1 receptor expression is rapidly down-regulated after IL 1 stimulation with internalization of the radiolabeled IL 1. We have now investigated factors that regulate IL 1 receptor expression. These studies have revealed that glucocorticoids and prostaglandins up-regulate IL 1 receptor expression on human B lymphocytes and fibroblasts. We have established the functionality of glucocorticoid induced IL 1 receptor on B cells by showing that IL 1 induces considerable protein phosphorylation at serine residues of a 65 kDa cytosolic protein in glucocorticoid treated human peripheral blood mononuclear cells (PBMC). This 65 kDa protein, whose phosphorylation is selectively augmented by IL 1 stimulation in PBMC, has been purified to homogeniety and the amino acid sequence indicates that this 65 kDa protein is a novel protein.

We have also been studying the mechanism of the cytostatic/cytocidal effect of IL 1 on several types of tumor cell lines. We have observed that IL 1 synergizes with TNF and type I IFN in inducing terminal differentiation of the myelocytic M1 cell line into macrophages. We have also found that IL 1 selectively stimulates the production of a 25 kDa mitochondrial protein in A375 cells while being inhibited from growing by IL 1. The biochemical purification and amino acid sequence of this 25 kDa protein revealed that the 25 kDa protein is manganese type superoxide dismutase, suggesting the involvement of superoxide generation in tumor killing by IL 1. The better understanding of the regulation of IL 1 receptor expression and mechanism of IL 1 cytotoxicity should enable us to use IL 1, as a BRM, more effectively.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

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OBJECTIVES

It is the goal of this project to understand the pathophysiological role of interleukin 1 and pursue the possible therapeutic application of IL 1. The problems being studied; include biological effects of IL 1 on a variety of cells and the molecular and biochemical analysis of IL 1 production, biological characterization of the IL 1 receptor, signal transduction mechanism, and the regulation of gene expression by IL 1.

METHODS EMPLOYED

Receptor binding assays were performed using 125I-labeled recombinant human IL 1α as previously reported. To identify phosphoprotein(s) whose phosphorylation is augmented by IL 1 stimulation, normal human PBMC were first cultivated with [32P]-orthophoshate and stimulated with recombinant IL 1α. Phosphoproteins were analyzed by SDS-PAGE and two dimensional gel electrophoresis followed by autoradiography. To purify 32P-labeled phosphoprotein, HPLC was used. To identify IL 1 inducible proteins, cells were labeled with 35S-Met after the culturing the cells with or without IL 1. Amino acid sequences were determined by the Edman degradation method. Northern and Western blotting analyses and recombinant DNA techniques were also utilized for the study of the production of IL 1α and IL 1β and expression of recombinant precursor IL 1α.

MAJOR FINDINGS

I. Study of the Production of IL 1

We previously reported that IL 1 molecules can be detected intracellularly as early as 30 min and extracellularly within 1 hr after stimulation of human monocytes with lipopolysaccharide. Surprisingly, most of the intracellular precursors of IL 1α and IL 1β were located in the cytosol fraction. [32P]-orthophosphate labeling of LPS-stimulated human monocytes showed that precursor site of IL 1α is more heavily phosphorylated at Ser residues than that of IL 1β. In vitro phosphorylation of synthetic peptide analogues of the precursor IL 1α suggested that C-AMP dependent protein kinase phosphorylates precursor IL 1α. Using E. coli expressed recombinant human precursor IL 1α, the effect of phosphorylation on the biological activity and receptor competing activity of precursor IL 1α have been examined. We observed that precursor IL 1α

possessed both thymocyte comitogenic activity and receptor competing activity, and that the phosphorylation by cAMP kinase did not change the comitogenic activity of precursor IL 1α. The effect of phosphorylation on IL 1 processing will further examined.

II. Regulation of IL 1 Production

Molecular analysis of the suppression of IL 1 production by adherent human mononuclear cells by a glucocorticoid hormone (prednisolone) was also performed. From 10 -5 to 10 -8M prednisolone inhibited both IL 1α and IL 1β production by LPS stimulated adherent human peripheral blood mononuclear cells in a dose dependent fashion as assessed by Western blotting of cell-associated IL 1, and a thymocyte comitogenic bioassay. IL 1α and IL 1β mRNA levels were concomitantly suppressed by glucocorticoid treatment as determined by Northern blotting analysis. Our data suggest that the suppression of IL 1 activity is due to decreased production of IL 1 proteins and mRNA, and is therefore probably regulated at the transcriptional level.

III. Regulating IL 1 Receptor Expression

We previously reported that IL 1 down-regulates its own receptor expression. This is accompanied by internalization of receptor bound IL 1. Since glucocorticoids and prostaglandins directly regulate expression of many genes, we studied the in vitro effect of glucocorticoids (GC) on IL 1 receptor expression of human peripheral blood mononuclear cells (PBMC). In physiologic and pharmacological concentration ranges, GC increased the specific binding of 125I-labeled human recombinant IL 1α to PBMC. This enhancement was specific for GC, since other steroid hormones, such as progesterone, 17-beta estradiol and testosterone failed to elevate the binding of 125I-IL 1α to PBMC. The effect was time-dependent, with maximal effect occuring 6 hr after treatment and dose-dependent with half maximal effect elicited by 100 nM prednisolone. Scatchard plot analysis indicated that 125I-IL 1α binding increased from about 100 IL 1 receptors per cell to 2 x 10 to the 3rd receptors per cell without a major change in affinity (kd = 2.6 x 10 -10M). The subpopulation of PBMC induced by GC to express higher levels of IL 1 receptors consisted predominantly of B lymphocytes, but not T lymphocytes, large granular lymphocytes, or monocytes. Glucocorticoids also induced the expression of IL 1 receptors on some other cell types, including normal human dermal fibroblasts and the human large granular lymphocyte cell line, YT. Since cycloheximide and actinomycin-D inhibited the induction of IL 1 receptor by GC, synthesis of both new RNA and protein seems to be required for IL 1 receptor induction. These findings may be physiologically relevant since IL 1 augmented polyclonal immunoglobulin production by monocyte depleted B lymphocytes only in the presence of glucocorticoids. Furthermore, polyclonal B lymphocyte activation could be inhibited by anti-IL 1β antibody, suggesting that glucocorticoid induced IL 1 receptors are functional and IL 1 mediates B cell differentiation by glucocorticoids.

The in vitro effects of prostaglandins, glucocorticoid hormones (GC) and IL 1 on interleukin 1 receptor (IL 1R) expression of human dermal fibroblasts were also investigated. PGE1, PGE2 and GC each increased the specific binding of 125I-IL 1α to fibroblasts. Scatchard analysis indicated that the number of IL 1R increased from 1.6 x 10 to the 3rd on control cells to 5.4 x 10 to the

3rd on PGE2 treated cells, and to 5.7 x 10 to the 3rd on GC treated cells without a significant change in the high binding affinity (Kd 4.6 x 10 -10M). The combination of PGE2 and GC increased the number of IL 1R in an additive manner to 11 x 10 to the 3rd per cell. Forskolin, an activator of adenylate cyclase, as well as analogues of cAMP also up-regulated IL 1R expression by fibroblasts. Cycloheximide and actinomycin-D abolished the effect of PGE 2 and GC on IL 1R induction. Exposure of fibroblasts to IL 1 at 37 C resulted in rapid receptor down-regulation (75%) by 3 hrs. The binding capacity of fibroblasts for 125I-IL 1α was restored to control levels by 16 hrs after IL 1 treatment and subsequently increased to about two-fold over control levels by 48 hrs. This late enhancement of IL 1 binding activity was inhibited by indomethacin. Consequently, the stimulatory effect of IL 1 on IL 1 receptor expression is presumably mediated by prostaglandins and intracellular cAMP signals.

IV. Signal Transduction by IL 1

We have further established the functional capability of such glucocorticoid induced IL 1 receptors by showing that they transduce increased protein phosphorylation. Carrier-free recombinant human IL 1α specifically induced phosphorylation of an acidic 65 kDa protein (pp 65) at serine residues within 5 min in glucocorticoid treated PBMC. Fractionation of IL 1 stimulated PBMC, after treatment with prednisolone showed the pp 65 to be located in the cytosol, suggesting that pp 65 is not the IL 1 receptor itself. Both unphosphorylated and phosphorylated 65 kDa proteins have been purified to homogeneity by sequential chromatography including Sephacryl S-200 gel filtration, HPLC anion exchange and HPLC hydroxyapatite from the cytosol of IL 1 stimulated steroid treated PBMC. Amino acid composition analyses of unphosphorylated and phosphorylated 65 kDa protein gave identical results. Amino acid sequence analysis of this protein showed that pp 65 has a novel protein sequence. cDNA cloning for pp 65 is in progress. In conclusion, these data suggest that the glucocorticoid induced IL 1 receptor is functional and either contains or is associated with a serine kinase which phosphorylates a novel cytosolic 65 kDa protein.

V. Anti-tumor Effects of IL 1

We investigated the effect of combinations of cytokines known to be cytostatic for some tumor cells, i.e. IL 1α, interferon β1 (IFN β1) and tumor necrosis factor (TNF), on the in vitro growth and differentiation of the mouse myeloid leukemic M1 cell line. IL 1α, IFN β1 and TNF by themselves partially inhibited the growth of M1 cells. Treatment of cells with a mixture of any two of the three cytokines resulted in additive or greater inhibition of growth. None of these cytokines by themselves induced differentiation of M1 cells as assessed by increased expression of Fc receptors (FcR), stimulation of phagocytic activity and by morphological criteria. However, as little as 1 U/ml IL 1α in conjunction with IFN β1 or TNF induced differentiation in addition to inhibiting the growth of M1 cells. The combination of IFN β and TNF did not induce differentiation, although the growth of the cells was markedly inhibited. Both TNF and LPS induced the in vitro production of IFN activity by M1 cells. Furthermore, the induction of differentiation of M1 cells by a combination of IL 1α with either IFN β 1, TNF or LPS was inhibited by antibody against mouse

IFN β1, but not by anti-IFN α. Therefore, it appears that IFN β1 provides one of the two required signals for differentiation of M1 cells by these combinations of stimulants, the other being IL 1. Furthermore, the cytostatic effect of TNF by itself on M1 cells was also partly blocked by anti-IFN β1 antibody, suggesting that IFN β1 is also involved in the growth inhibitory effect of TNF for M1 cells. In contrast, the cytostatic effect of IL 1 on M1 cells was not blocked by anti-IFN β1 antibody. In conclusion, both the cytostatic and differentiative effect of TNF appear to be mediated by IFNβ1. Thus, the combination of IL 1 and IFN β1 or inducers of of IFN β1 resulted in terminal differentiation of M1 cells.

IV. Study of the Mechanism of Cytocidal Effect of IL 1 on Human Melanoma Cells

IL 1 is cytocidal for several human tumor cells in vitro. The cytocidal effect of IL 1 on human melanoma A375 cells occurs after 48 hr treatment with IL 1. In order to identify the intracellular events preceding cell death, we first examined the effects of IL 1 on the proto oncogene expression and ornithine decarboxylase (ODC) activity in A375 cells. IL 1 rapidly and transiently induced c-fos mRNA expression at 30 min, but c-myc mRNA levels begin to decrease after 3 hrs and ODC begin to decrease after 6 hrs of exposure to IL 1. We also examined the effects of IL 1 on general protein synthesis in A375 cells. Cultured A375 cells were treated with recombinant human IL 1α and radiolabeled with 35S-methionine. Cell extracts were analyzed by SDS-PAGE. Selective and marked induction of a 25 kDa protein (p25) was observed within 12 hrs in IL 1 treated melanoma cells. In order to localize the p25, cells were homogenized and fractionated by differential centrifugation. The p25 was located exclusively in the 10,000xg pellet (mitochondria enriched fraction). The p25 was purified from the mitochondrial enriched fraction by sequential chromatography on DEAE Sephacel and HPLC anion exchange. This yielded purified p25 that was homogeneous on SDS-PAGE and permitted determination of the amino acid sequence. The amino acid sequence of purified p25 was identical to that of manganese superoxide dismutase (Mn-SOD). Although the induction of Mn-SOD precedes the cell death, it remains to be established whether or not the induction of this protein is causally involved in cell death following IL 1 administration. SOD induction by IL 1 may also explain the radiation protection activity of IL 1.

SIGNIFICANCE

Identification of stimulants that up-regulated IL 1 receptor expression may lead us to a further understanding regarding the regulation of IL 1 receptor expression under physiological conditions. This may also lead us to purify the IL 1 receptor and construct better cDNA libraries for the cloning of the cDNA for the IL 1 receptor. Identification of an intracellular phosphoprotein whose phosphorylation is selectively augmented by IL 1 stimulation has established that the IL 1 receptor is associated with a serine kinase. The synergistic effect of IL 1 with TNF and type I IFN on the growth of tumor cells encourages us to further screen other types of tumor cells and determine the effect of IL 1 on tumor cells in vivo. The induction of SOD by IL 1 may also explain the mechanism of radioprotection activity of IL 1 and this finding also encourages us to try therapeutic applications of IL 1 in regard to protection of bone marrow cells and tissues from radiation therapy.

PROPOSED COURSE

One major goal is the purification and cDNA cloning of human IL 1 receptors. The establishment of the biological significance of the intracellular precursor IL 1α and β and the role of the phosphorylation of these proteins will be studied out using recombinant DNA techniques. The study of signal transduction upon IL 1-IL 1 receptor interaction, the identification of intracellular biochemical events after IL 1 stimulation and the identification of a kinase which phosphorylates this protein has been extensively carried out. Presently, the cDNA cloning of the pp 65 is being undertaken. The in vitro and in vivo mechanism of antitumor and radioprotective effects of IL 1 will be pursued in collaboration with several other groups.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09283-03 LMI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Regulation of Gene Expression in Immune Effector Cells

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

PI:	H. A. Young	Expert	LMI, NCI
Others:	S. Sakamoto	Visiting Fellow	LMI, NCI
	E. Kovacs	Biotechnology Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	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 project is divided into three parts: 1) control of specific lymphokine (i.e. interferon gamma) gene expression; 2) the pattern of lymphokine gene expression during maturation of immune effector cells (i.e. LAK cells) and 3) the role of T cell receptor DNA structure during the growth and differentiation of immune effector cells (i.e. T cells, LGL, B cells and monocytes).

1) We have found that deletion of specific regions of human interferon gamma genomic DNA can significantly effect the expression of this gene after transfection of these constructs into cells of a heterologous species. In addition, we have observed that the genomic DNA sequences 5' to the coding region of the gene, the region of genomic DNA generally found to be sufficient for expression of most cellular genes, is required but not sufficient for expression of human interferon gamma.

2) Analysis of lymphokine mRNA gene expression during in vitro LAK cell development has revealed that there are significant differences in lymphokine mRNA levels when LAK cells from different individuals are compared. Nevertheless, certain consistent patterns of lymphokine mRNA expression were observed. Specific lymphokine mRNAs were detected early in LAK cell in vitro growth while other lymphokine and cytokine mRNAs were observed to appear only during the latter stages of LAK cell generation.

3) Comparison of the methylation pattern of the T cell receptor beta chain gene (T beta) in different lymphoid populations revealed distinct differences between these cell populations. Hypomethylation of the gene was found to correlate with gene expression and may be important in the recombination events with generate specific T beta gene products. In addition, large granular lymphocytes (LGL) were found to have a very diverse pattern of methylation of this gene, indicating that this type of cell may be composed of multiple subpopulations of cells with distinct T beta methylation patterns.

GPO 914-916

PROJECT DESCRIPTION

PERSONNEL

Howard A. Young	Expert	IS LMI NCI
Shigeru Sakamoto	Visiting Fellow	IS LMI NCI
Elizabeth J. Kovacs	Biotechnology Fellow	IS LMI NCI

MAJOR FINDINGS

I. Control of Specific Lymphokine Gene Expression.

We have chosen interferon gamma as a model system for understanding how immunomodulatory cells control gene expression because only two cell types (T cells and LGL) are capable of producing this protein and it is produced in response to very specific stimuli. We have found that freshly isolated human peripheral blood T cells require two stimuli (e.g. PHA and IL 2 or PMA and PHA) before these cells produce IFN gamma, while LGL require only a single signal (e.g. IL 2, PMA, PHA) for the production of this protein. Furthermore, LGL can produce IFN mRNA as early as 30 min after IL 2 addition indicating that in these cells, the production of this protein is a direct response to external stimul. Thus, LGL appear to be primed differently than T cells for their response to external signals.

We have taken the approach of utilizing gene transfer technology in order to determine if specific regions of IFN gamma genomic DNA play a role in the transcriptional control of gene expression in response to specific stimuli. The human IFN gamma genomic DNA has been introduced into a mouse T lymphoblastoid line and mouse fibroblast cell lines. The mouse T cell line retains IL 2 receptors but does not require IL 2 for growth. After introduction of the human IFN gene into this cell line, a constitutive level of human IFN gamma protein was produced and production increased 3-5-fold in response to IL 2 and 30-50-fold in response to PMA. We have found that while both agents increase transcription of the transfected gene, PMA treatment also acts by stablizing the mRNA. Studies on the role of DNA structure in transcriptional control have resulted in the following observations: 1) the transfected gene appears to contain the same DNAse 1 hypersensitive sites as has been reported for the gene in Jurkat cells; 2) deletion of an intronic DNAse 1 hypersensitive site decreases transcriptional activity of the gene approximately 5-8-fold; 3) deletion of the middle two exons of the gene results in a 30-50-fold decrease in transcriptional activity of the gene and 4) the DNA region 5' to the TATA box is required but not sufficient for transcriptional activity of the gene. Thus, the role of specific DNA sequences in the control of human IFN gamma gene expression appears to be unique in comparison to other lymphokine genes.

Expression of the transfected gene in mouse fibroblasts is regulated by a different mechanism from that observed in the mouse T lymphoblastoid line. The gene is actively transcribed in mouse fibroblasts but no protein expression is observed unless the cells are treated with cycloheximide. This result indicates that either RNA processing, RNA transport from the nucleus or mRNA stability is involved in the control of expression of the transfected gene. We have found

that cycloheximide does not act to stabilize the mRNA since treatment of the cells with other protein synthesis inhibitors which do not have this effect, such as pactamycin and puromycin, also results in IFN protein expression. Parallel studies to those performed on T cells on the role of DNA structure on expression in mouse fibroblasts have indicated 1) deletion of the middle two exons of the gene results in decreased transcriptional activity but does not alter the effect of cycloheximide and 2) the DNA region 5' to the TATA box is not sufficient to promote transcription in the fibroblasts. These results indicate that specific cell types (in this case, fibroblasts) can effectively control specific gene expression by mechanisms unrelated to transcriptional control and studies utilizing transfection of human interferon gamma genomic DNA offer a unique model system with which to elucidate these mechanisms.

Our findings demonstrate that specific regions of human IFN gamma genomic DNA play important roles in the control of expression of this immunoregulatory molecule. Further analysis will permit more precise identification of those regions of genomic DNA structure involved in the transcriptional response to both PMA and IL 2 in T cells and the post-transcriptional control observed in fibroblasts.

II. The Pattern of Gene Expression During Maturation of Immune Effector Cells.

We have investigated the pattern of lymphokine gene expression during in vitro culturing of human lymphokine actived killer cells (LAK) with IL 2 in order to determine the role of lymphokine gene expression in the maturation of this mixed cell population. We undertook this project utilizing molecular technology because we believed it is not currently possible to measure a wide variety of lymphokine and cytokine activities in unfractionated culture medium and we want to determine if expression of specific lymphokine molecules can be correlated with in vitro and in vivo LAK activity. Utilizing Northern blot analysis of total cellular RNA, we have observed a general pattern of lymphokine gene expression in LAK populations from different individuals. Interferon gamma mRNA is seen during days 2-7 of culture while IL 1 mRNAs are seen most often during the first 1-3 days. In contrast to the IL 1 mRNA, TNF alpha, TNF beta and TGF beta mRNAs are seen late in the in vitro culturing, generally at days 5-7. This data indicates that a number of lymphokine and cytokine mRNAs can be detected during in vitro LAK cell generation and that the products of these genes presumably have an important role in tumor cell killing. Furthermore, experiments can be designed to determine 1) the specific role of these endogenous generated proteins in mediating LAK activity and 2) the effect of addition of exogenous proteins on the time course and levels of in vitro LAK activity.

III. The Role of T Cell Receptor Beta Chain Gene DNA Structure in the Growth and Maturation of Immune Effector Cells.

We have been investigating the relationship between human large granular lymphocytes and T cells at both the molecular and biochemical levels. As stated above, we have observed differences between these cell populations with regard to IFN gamma production in response to different external stimuli. Furthermore, we have previously found that LGL only produce a truncated, nonfunctional 1.0 kb mRNA from the T cell receptor beta chain gene while T cells

produce a functional 1.3 kb mRNA as well as a non-functional 1.0 kb transcript. Further studies have determined that CD3- LGL also fail to produce T cell receptor alpha and gamma chain mRNA transcripts. gene products in target cell recognition and killing. In order to determine a mechanism for potential differences in the utilization of the T cell receptor genes by LGL and T cells, we have compared the T cell receptor beta chain DNA structure in LGL, T cells, B cells and monocytes. Utilizing the restriction enzymes, Msp 1 and Hpa 2, we have observed that the T cell receptor beta chain gene in T cells is substantially hypomethylated while the gene in LGL is significantly more methylated, with the DNA pattern indicating that CD3- LGL may represent a diverse, mixed population of cells. Furthermore, we have found that the gene in B cells and monocytes is almost completely methylated but homogenous in contrast to that seen in LGL. This data indicates that methylation of specific DNA sequences correlates with active gene transcription and rearrangement and may be an important controlling factor in both of these processes involving the T cell receptor beta chain gene. Furthermore, the methylation or hypomethylation of specific regions of this gene may be involved in the differentiation of precursor cells towards a specific cell lineage. The use of the restriction enzyme Hpa II in combination with other restriction enzymes to generate a specific restriction enzyme pattern of this gene may also provide a marker for malignant cell populations of unknown origin.

In summary, this laboratory has continued to devote its efforts toward elucidating the mechanisms by which cell populations involved in cellular immunity regulate specific gene expression in response to external stimuli. We have focused, in large part, on determining the role of DNA structure in controlling gene expression and cell differentiation and maturation. It is anticipated that this approach will continue to yield valuable information on how extracellular events result in specific responses at the genetic level.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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

Role of Interleukin 1 in the Immune Response

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

PI:	S. K. Durum	Sr. Staff Fellow	LMI, NCI
Other:	L. Takacs	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

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Immunobiology Section

INSTITUTE AND LOCATION

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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.)*

Interleukin 1 (IL 1) is a soluble mediator that promotes several phases of the immune response. For the past year, we have studied various features of the production of this molecule. We previously observed that one T cell clone produced IL 1 and have extended this observation; it is now clear that many T cell clones produce IL 1, and utilize this mediator, in an autocrine fashion, for their own growth. These were quite unexpected findings, since IL 1 has been generally viewed as an exogenous cofactor for T cell replication.

Most studies of lymphokines have been conducted in vitro. It is important to perform in vivo studies to determine how lymphokines arise and are utilized. We have analyzed IL 1 gene expression in vivo by in situ hybridization for IL 1 mRNA in single cells in sections of mouse tissues. We observe that cells producing IL 1 mRNA are found chiefly in lymphoid organs: thymus, spleen and lymph nodes; uterus also contains frequent IL 1 mRNA producing cells. Surprisingly, no intentional immune or inflammatory stimuli were required to induce IL 1 gene expression in these organs.

A "membrane IL 1" has recently been described, and this protein represented an IL 1-like biological activity on the surface of fixed cells. We have investigated the biochemical nature of this membrane IL 1 and show that it is the pro-IL 1 α molecule, probably associated with a cell surface lectin that recognizes mannose residues on pro-IL 1. This lectin may be involved in exporting IL 1 from the cell interior, since IL 1 lacks the signal peptide usually associated with cell surface and secreted molecules.

PROJECT DESCRIPTION

PERSONNEL

Scott K. Durum	Senior Staff Fellow	IS LMI NCI
Laszlo Takacs	Visiting Fellow	IS LMI NCI

OBJECTIVES

We have pursued the following questions regarding the role of IL 1 in immune responses:

1. We previously showed that a T cell clone produced IL 1 - we next asked: is this a general property of T cells, and does T cell IL 1 have a functional role for the T cell producing it?

2. Are IL 1 genes active in vivo during processes of immunity, inflammation and development?

3. "Membrane IL 1" was previously described by others as a biological activity that resembled IL 1 but was present on fixed macrophages - what is the biochemical relationship of membrane IL 1 to the well-characterized soluble IL 1 molecules?

METHODS EMPLOYED

IL 1 was detected by bioassay using the D10.G4.1 clone. Proliferation of various T cell clones was assessed by incorporation of radiothymidine. Anti-IL 1 was produced in rabbits against recombinant murine IL 1. In situ hybridization for IL 1 gene expression was performed using 32P-labeled cDNA probes on frozen tissue sections. Membrane IL 1 from LPS-activated macrophages was isolated by immunoprecipitation and analyzed by SDS-PAGE.

MAJOR FINDINGS

1. We have extended our previous findings regarding T cell IL 1 to many murine and human T cell clones; these cells produce IL 1 following activation by antigen presenting cells or lectins. This T cell IL 1 may perform several functions, one function being an autocrine growth factor, since anti-IL 1 blocked T cell proliferation. It appears that T cell IL 1 is initially produced in an inactive form, requiring proteolytic cleavage (by an "IL 1-activator") to become active.

2. In situ hybridization was used to visualize mRNA for IL 1 in individual cells in tissue sections. Having examined many murine organs, we conclude that IL 1 mRNA producing cells are predominantly located in lymphoid organs and that no requirement for (intentional) exogenous stimulation by antigens or inflammatory agents was observed. IL 1 mRNA producing cells were detected in thymus at the cortico-medullary junction, in sinuses of lymph nodes, and in spleen in the marginal zone and red pulp; uterus also contained frequent cells in subepithelial connective tissue.

3. Membrane IL 1 has been analyzed biochemically. We conclude that it consists of the 33 Kd pro-IL 1α peptide, that it is glycosylated and is associated with the plasma membrane via a lectin-like interaction specific for the sugar mannose. Thus, IL 1 is anchored to the membrane by a mechanism that is probably similar to that of lysosomal hydrolases, which are also bound to a cell surface lectin.

SIGNIFICANCE

1. Our observation that T cell clones produce and utilize their own IL 1 contradicts the paradigm that T cells require IL 1 from exogenous sources. We observe however that normal unprimed T cells do not produce their own IL 1 and presumably require exogenous IL 1. Clones may thus represent a type of memory T cell capable of continuous self-renewal.

2. Our study of IL 1 gene expression in vivo by in situ hybridization represents the first analysis of this type for any lymphokine. One of the most intriguing findings is the expression of IL 1 genes in thymus, which may signify a role for IL 1 in T cell development.

3. The existence of a plasma membrane form for IL 1 would not have been predicted from the cDNA sequence - there is no signal peptide (for crossing membranes) or a predicted transmembrane segment (for anchoring). Our observations suggest the hypothesis that IL 1 is bound to a lectin on the cell surface and this in turn implies that the lectin may be involved in transporting IL 1 from the cytosol to the exterior of the cell.

PROPOSED COURSE

1. T cell IL 1 will be approached from several directions. Why is IL 1 produced by T cell clones but not by most normal T cells - does cloning select for a subset of T cells capable of IL 1 production or does cloning activate IL 1 genes? We will look for repressors of IL 1 gene expression in T cells that do not produce IL 1. We will look for T cells producing IL 1 in vivo using in situ hybridization. We will extend our analysis of IL 1 production in T cell clones to include other subsets of helper clones (types I and II of T. Mosmann) and cytotoxic clones (from M. Sitkovsky).

2. In situ hybridization for IL 1 gene expression will be extended to embryonic tissues, to normal human tissues and to pathological specimens in man and mouse. It will be particularly important to analyze tumor infiltrates and tissue specimens from chronic inflammatory diseases (e.g. arthritic joints). We must also adapt our techniques to permit identifying the cell type expressing IL 1 mRNA and we aim to be able to use cell specific antibodies for this purpose, although our present in situ hybridization technique destroys most protein epitopes recognized by antibodies.

3. Having shown that membrane IL 1 is anchored by a lectin-like interaction, we will analyze whether this lectin is responsible for transporting IL 1 from the cytosol; if so, this will represent a novel protein secretory mechanism, and we will examine other proteins for secretion via this mechanism. We will also extend these studies to human macrophages and other cell types and to the other major IL 1 gene product (IL 1β). The postulated lectin (to which IL 1 appears to be bound) will be characterized.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09251-05 LMI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Interactions of Human Retroviruses with Hematopoietic and Adherent Cells

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

PI:	F. W. Ruscetti	Senior Investigator	LMI, NCI
Others:	G. K. Sing	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

Lymphokine Section, LMI, NCI (W. Farrar); Laboratory of Experimental Immunology, NCI (J. Ortaldo); Upstate Medical Center (B. Poiesz); National Institute of Mental Health, NIH (C. Pert).

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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.)*

Acquired immunodeficiency syndrome (AIDS) is associated with a viral (HTLV-III) mediated progressive depletion of the helper/inducer T4+ T cell subset, whereas acute T cell leukemia is associated with viral (HTLV-I) mediated growth of the same T cell subset. We have recently demonstrated that large granular lymphocytes (LGL) with natural killer activity have been able to spontaneously lyse several types of virus infected target cells. IL 2 activated T cells and NK cells were cytolytic for HTLV-I infected target cells. However, only large granular lymphocytes demonstrate significant spontaneous activity against HTLV-I and HTLV-III targets. In addition, the differentiation antigen T4 is present on the helper/inducer T cell subset, which is responsible for the humoral regulation of immune reactivity and hematopoietic development. In AIDS, this lymphocyte subset is selectively depleted by infection with HTLV-III virus which apparently uses the T4 antigen complex on the T-cell surface as a viral receptor. Since patients with AIDS frequently develop CNS symptoms, we investigated sites of entry of HTLV-III in monkey brains. Autoradiographic visualization revealed T4 antigen on thin coronal sections of squirrel monkey brain in a strikingly heterogeneous pattern with clustering in the same emotion-mediating nodal points of the brain that are usually enriched with neuropeptide receptors. Other T lymphocyte associated antigens such as T8 were not found in the brain. These data suggest that direct infection of some brain cells with HTLV-III occurs via a mechanism involving the T4 antigen similar to that in lymphocytes. Finally, based on our findings of the presence of receptors for CD4, and pathogenic viruses in the immune and central nervous system, we have studied the ability of neuronal peptides to inhibit HTLV-III infection. Several synthetic peptides based on known sequences of neuronal peptides were found to act as possible competitive analogues against HTLV-III infectivity.

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

PROJECT DESCRIPTION

PERSONNEL

Francis W. Ruscetti	Senior Investigator	LS LMI NCI
Garwin K. Sing	Visiting Fellow	LS LMI NCI

MAJOR FINDINGS

I. Cytotxic Effector Mechanisms are Active Against Human Lymphocytotropic Viral Infected Cells

Acquired immunodeficiency syndrome (AIDS) is associated viral (HTLV-III/ LAV) 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 lymphoid cell lines and fresh lymphocytes was explored. Normal lymphocytes (T cells and LGL), with and without pretreatment with recombinant interleukin 2 (IL 2), as well as monocytes, with and without pretreatment with gamma interferon were employed as effectors. Both IL 2-activated T cells and NK cells were cytolytic for HTLV-I infected targets. However, only LGL demonstrated significant spontaneous activity against HTLV-I infected targets. Similarly, LGL showed spontaneous cytolytic activity against HTLV-III/LAV infected targets and this cytotoxicity was considerably augmented by IL 2. In contrast, T cells and monocytes were unable to lyse HTLV-III/LAV targets and only minimal activity was induced by activation. LGL cells, B cells, and monocytes could be infected in vitro by high titers of HTLV-III/LAV. However, levels of reverse transcriptase found in these cultures were significantly lower than the levels in T cell cultures. In contrast, only T cells 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), but not from infection by HTLV-III/LAV. Collectively, these results indicate that NK cells may play a role in protecting cells against HTLV infection, but their effector role may be abrogated during HTLV-III/LAV infection due to susceptibility to this virus. Also, these data support the concept that other cells besides T4+ T cells can serve as reservoirs of HTLV-III/LAV replication.

II. T4 Antigen, the Cell Surface Receptor for HTLV-III/LAV is Found in Specific Areas of the Brain

Monoclonal antibodies are available that separate mature human T cells into functional subsets. The differentiation antigen T4 is present on the helper/inducer T cell subset, which is responsible for the humoral regulation of immune reactivity and hematopoietic development. In the acquired immunodeficiency syndrome (AIDS), this lymphocyte subset is selectively depleted by infection with HTLV-III/LAV virus which apparently uses the T4 antigen complex on the T-cell surface as a viral receptor. Here, we show that autoradiographic visualization of the T4 antigen on thin coronal sections of squirrel monkey brain reveals a

strikingly heterogeneous pattern with clustering in the same emotion-mediating nodal points of the brain that are usually enriched with neuropeptide receptors. Immunoprecipitates of radioiodinated cell membranes prepared from primate brain indicate that an antigen very similar to T4 antigen is present on brain as well as T4+ T-lymphocytes. Other T lymphocyte-associated antigens such as T8 were not found in the brain. Since patients with AIDS frequently develop complications of the central nervous system and HTLV-III/LAV sequences have been found in the brain, these data suggest that direct infection of some brain cells with HTLV-III/LAV occurs via a mechanism involving the T4 antigen complex similar to that proposed for lymphocyte infection. In view of the brain sites found to express the T4-like antigen, the behavioral changes and mood shifts observed in AIDS patients may result from localized viral replication.

III. Octapeptides Deduced From the Neuropeptide Receptor-like Pattern of Antigen T4 in Brain Potently Inhibit Human Immunodeficiency Virus Receptor Binding and T-cell Infectivity

The differentiation antigen T4, present on the helper/inducer subset of T lymphocytes, is thought to serve as the receptor for the human immunodeficiency virus (HIV). We find that a 60-kDa protein, immunoprecipitable by monoclonal antibody (mAb) OKT4, is present on membranes from human brain as well as human T cells. Furthermore, the radioiodinated HIV envelope glycoprotein [125I-labeled gp120 (125I-gp120)] can be specifically covalently affixed to a molecule present on rat, monkey, and human brain membranes to yield a complex that is indistinguishable from that formed on human T cells. T4 antigen has been studied on unfixed squirrel monkey, rat, and human brain sections by autoradiography using the mAb OKT4. A highly conserved neuroanatomical pattern has been demonstrated, suggesting an analogous organization in these three mammalian brains. Furthermore, the localization of 125I-gp120 receptor binding appears similar to that of T4 and is highly reminiscent of patterns for many previously characterized neuropeptide receptors. A computer assisted analysis of gp120 suggested that a previously unremarkable octapeptide sequence within the gp120 protein, which we have synthesized and termed "peptide T," may play an important role in HIV attachment. Thus, peptide T and three rationally designed peptide analogs, each with a systematic amino acid substitution, potently inhibit specific 125I-gp120 binding to brain membranes. Additionally, when tested in a viral infectivity assay, these peptides show the same rank order and similar absolute potency to block HIV infection of human T cells. Thus, peptide T may provide a useful pharmacological or immunological basis for the control and treatment of AIDS.

IV. cis-Acting Transcriptional Regulatory Sequences in the Gibbon Ape Leukemia Virus (GALV) Long Terminal Repeat

Gibbon ape leukemia viruses (GALV) are a group of retroviruses which have been associated with hematopoietic neoplasms in primates. Two of the viruses, GALV-SEATO and GALV-San Francisco (GALV-SF), are associated with myeloid and lymphocytic leukemias, respectively, in apes. Using an assay based on the transient expression of the bacterial gene chloramphenicol acetyltransferase (CAT), we examined the transcriptional activity of GALV-SEATO and GALV-SF. The results suggest that high level expression of GALV is due primarily to cis-acting enhancer sequences. Sequence delineation analysis of GALV-SEATO showed the GALV-SEATO enhancer sequences to be located with a 45-bp tandem repeat in GALV-SEATO,

GALV-SF, which has two-to five-fold transcriptional activity, contains only a single copy of the 45-bp element with a 6-bp differences from those in the GALV-SEATO enhancer element. The 45-bp element is highly homologous to sequences within the LTRs of several murine leukemia viruses but has not been examined for enhancer function in these retroviruses. Expression of GALV was not restricted to hematopoietic cells but was extraordinarily high in MLA 144 cells, a gibbon ape T-cell line known to be infected with GALV-SF. However, expression of constructs containing the CAT gene directed by GALV SEATO LTR sequences was similar in uninfected and GALV-infected fibroblasts, indicating the lack of virally encoded or virally induced trans-activating factors capable of increasing expression in these cells.

V. Interaction of Human Cytomegalovirus with HTLV and HIV-infected Lymphocytes

Direct impairment of human T cell-mediated immune function by viral infection is most aptly demonstrated by the acquired immunodeficiency syndrome (AIDS), which is the result of human immunodeficiency virus (HIV)-mediated depletion of a helper/inducer CD4+ T cell subset. In contrast, the other known human retroviruses, HTLV I and II are associated with uncontrolled growth of the same subset, which may result in acute T cell leukemia. Amongst the other human viruses also known to infect T helper cells are some members of the Herpes virus family, particularly human cytomegalovirus (HCMV). This virus is also known to cause immunosuppression, and is commonly associated with AIDS-related deaths. Nevertheless, the relationship between HCMV and HTLV or HIV remains obscure, neither is it known whether the viruses can replicate simultaneously in the same cell. This present work was undertaken to investigate whether HCMV could infect T lymphocytes harbouring the HTLV I, II or HIV genome, and whether such superinfection with HCMV would result in a synergy so as to affect any characteristics of the host lymphocyte or affect the replication of either HCMV or the retroviruses. 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 preinfection 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 centres when assayed on permissive fibroblasts. In HTLV I and HTLV II-infected cell lines, cell viability and proliferation was not affected by infection with HCMV, and neither were there any significant differences in the expression of T cell-associated surface markers as determined by immunofluorescence studies. 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 IFN gamma 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. In 2 cell lines, H9 and MT2, viral mRNA also detected in mock-infected controls, suggesting that these cells came from

patients who has been exposed to HCMV previously. 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 long terminal repeat (LTR) sequences derived from a molecular clone of HTLV I fused to a bacterial chloramphenicol acetyltransferase gene (CAT). In contrast to the findings of others, 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 that for HTLV I at least, trans-activation of the HTLV I genome by HCMV can occur. The same studies are presently being conducted to see whether this can also occur for HTLV II and HIV.

IV. The Effects of Human Cytomegalovirus Infection on the Growth and Differentiation of Primary Human Bone Marrow Cells

Human cytomegalovirus has a particular propensity to produce severe morbidity and death in immunosuppressed patients, principally those with AIDS and allograft recipients as in bone marrow transplants. Indeed, HCMV infection remains the most common infectious cause of morbidity after bone marrow transplantation, and the virus has been shown to interfere with normal hematopoietic recovery in patients undergoing marrow-ablative chemoradiotherapy followed by autologous bone marrow transplantation. Although it has recently been shown that HCMV infects bone marrow fibroblasts and an OKT10+ subset of hematopoietic progenitors, little is know concerning the effects of this virus on normal hematopoietic development. This present work was undertaken to investigate the effects of HCMV challenge on normal human bone marrow progenitors to respond to recombinant human granulocyte-macrophage colony stimulating factor (rGM-CSF) and recombinant granulocyte colony stimulating factor (G-CSF). Although the virus had no effect on cell viability, the ability of these cells to proliferate in response to either GM- or G-CSF was inhibited following virus challenge. Although the 50% maximal dose of either colony stimulating factor was equal for both mock-infected and infected cells, maximal proliferation of mock-infected cultures was twice that of virus-infected cells as seen by 3H-thymidine incorporation, as was the degree of background proliferation. The ability to form colonies of all hematopoietic lineages in the presence of GM-CSF and erythropoietin was completely abrogated. Sensitivity to G-CSF was also inhibited, although a few colonies and clusters of cells still appeared by 14 days post-infection. To study the effects of the virus on cell differentiation, a number of human meyloid leukemia cell lines were challenged with HCMV and identified based on a number of morphological and cytochemical criteria. Of the lines tested thus far, the erythroleukemia line HEL and the myeloid-monocytic line KG-1 displayed characteristics of mature granulocytes in a small population of these cells. The degree of proliferation was also reduced in all lines tested. The viral genome was expressed although less than 20% of the cells were stained with immunofluorescent-labeled antibodies to the immediate-early antigen. These results demonstrate that HCMV is able to impair hematopoietic growth by interferring with progenitor cell sensitivity to colony stimulating factors. In a small proportion of the population, blocking of proliferation may be due to HCMV-induced differentiation into cells of the granulocytic series.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09254-05 LMI

PERIOD COVERED

October 1, 1986 to September 20, 1987

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

Biochemical and Molecular Mechanisms of Growth Factor Modulated Proliferation

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

PI:	W. L. Farrar, Jr.	Senior Staff Fellow	LMI, NCI
Others:	A. H. Bellan	Visiting Fellow	LMI, NCI
	S. W. Evans	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

National Institute of Mental Health, NIH (C. Pert); Division of Cancer Etiology, NCI (J. Cleveland); PRI, NCI-FCRF (L. Arthur).

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Lymphokine Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

We have continued investigations which focus on identifying the biochemical signals and gene expression induced by lymphoid and myeloid growth factors. Interleukin 2 (IL 2), which stimulates the growth and differentiation of lymphoid cells, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF) and interleukin 3 (IL 3), all which stimulate myeloid cell differentiation, were investigated. The studies have examined the early phosphorylation events induced at the membrane by each of the growth factors as well as the regulation of mRNA accumulation of at least sixteen genes. The biochemical studies included: 1) activation of specific kinases; 2) identification of kinase substrates and functional modification of the identified substrate; 3) modulation of the adenylate cyclase system; 4) activation of GTP-binding protein activity and; 5) synthesis of Ap4A nucleotides. In addition, we have examined the regulation of stable mRNA accumulation for specific genes in response to IL 2 and the above CSFs as well as cyclic AMP, an antigrowth signal for lymphoid and myeloid cell growth. We have also examined the effects of cyclic AMP analogues on IL 2 and CSF stimulated gene expression and protein synthesis.

As a second major effort the laboratory has examined receptors common in the immune and central nervous systems. We have found the receptors for the cytokine interleukin 1 and human immunodeficiency virus (CD4) in brain to be indistinguishable from that observed in immune cells. In situ hybridization with cDNA probes recognizing IL 1 mRNA have localized IL 1 mRNA accumulation in normal brain. Northern analysis revealed that IL 1β mRNA is of a similar maturation size as that observed from monocytes. CD4 mRNA is, however, truncated in human brain as compared to T lymphocytes.

GPO 914-916

PROJECT DESCRIPTION

PERSONNEL

William L. Farrar, Jr.	Senior Staff Fellow	LS LMI NCI
Stuart W. Evans	Visiting Fellow	LS LMI NCI
Annick H. Bellan	Visiting Fellow	LS LMI NCI

OBJECTIVES

The laboratory has developed research programs in two areas: 1) biochemical and molecular mechanisms of lymphoid and myeloid growth, and 2) receptors and chemical signals common to the immune and central nervous systems.

MAJOR FINDINGS

I. Biochemical and Molecular Mechanisms of Lymphoid and Myeloid Growth

We have identified a number of biochemical events following the interaction with a lymphoid growth factor (IL 2) and a myeloid growth factor (IL 3) with their specific high affinity receptors. These include: 1) activation of at least two distinct kinase systems; 2) inhibition of basal and hormonal adenylate cyclase activity; 3) development of GTP-binding proteins associated with ligand-receptor interaction; 4) synthesis of a phosphorylated diadenylate which facilitates DNA polymerase alpha activities; and 5) phosphorylation of 40S ribosome S6 protein which regulates the rate of protein elongation.

We have characterized two unique kinases which are activated by IL 2 and other CSFs. IL 2, IL 3, and G-CSF apparently stimulate the activation of a phospholipid-Ca 2+-dependent kinase, protein kinase C. A substrate of this kinase has been identified in lymphoid and myeloid tissue and is a 68 kD protein phosphorylated on serine residues. The other kinase activity identified was a Mg 2+-dependent activity which phosphorylates the 40S ribosomal S6 protein. Phosphorylation of this protein was positively correlated with the ability of ribosomes to elongate protein chains.

IL 2 stimulates the accumulation of a highly phosphorylated derivative of ATP, diadenosine tetraphosphate (Ap4A). The accumulation of Ap4A correlated with the ability of lymphocytes to undergo DNA synthesis.

The transcriptional regulation of several genes by lymphokines and their target tissues has been examined. These results were compared with direct kinase activators which either promote (phorbol esters) or inhibit (cyclic AMP) cellular proliferation to the growth factors. IL 2, IL 3, GM-CSF and G-CSF all induce a similar program of mRNA expression for the nuclear proto oncogenes, c-fos, c-myc and c-myb in their respective cell lines. Phorbol esters stimulated the same temporal expression of proto-oncogenes, and the anti-growth kinase activator inhibited the expression of c-myc. The anti-growth kinase activator (cAMP) also inhibited the translation of abundant mRNAs, suggesting multiple molecular sites of cAMP anti-growth activity.

IL 2 and CSFs were also shown to stimulate gene expression and protein activity of an important enzyme for DNA synthesis, ornithine decarboxylase. Phorbol esters also stimulated this gene transcription whereas cAMP suppressed mRNA accumulation of this gene.

II. Common Receptors to the Immune and Central Nervous Systems

We have identified two unique receptors in brain previously identified in the immune system. The receptor for the monokine IL 1 was detected using 125I-IL 1 binding to rat brain sections. Cross-linking studied revealed the receptor size to approximate that seen in lymphoid and fibroblast tissues. The receptor for human immunodeficiency virus (HIV) was also detected in rodent and human brain using a monoclonal anti-receptor antibody and 125I-gp 120 virus envelope protein. Consequently, a quantitative radioligand assay using 125I-gp 120 has been developed for CD4+ lymphocyte lines. We are attempting to characterize the envelope protein-receptor interaction and develop analogs of the receptor and envelope protein which may be useful in the therapeutic development based on inhibition of the virus-cell interaction.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09264-05 LMI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Regulation of Normal and Neoplastic Hematopoietic Cell Growth: Role of BRMs

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

PI:	F. W. Ruscetti	Senior Investigator	LMI, NCI
Others:	M. C. Sparks	Guest Researcher	LMI, NCI
	G. K. Sing	Visiting Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Immunology, NCI (J. Ortaldo); Immunobiology LMI, NCI (H. Young); PRI, NCI-FCRF,(J. Rossio, J. Keller), Food and Drug Administration (E. Bonvini).

LAB/BRANCH

Laboratory of Molecular Immunoregulation

SECTION

Lymphokine Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	.75	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Binding of specific growth factors to their specific receptor is a prerequisite for specific cell types to grow. This laboratory is interested in studying the events necessary for the growth and differentiation of normal and neoplastic hematopoietic cells. As a model, recombinant interleukin 2 (IL-2) has been used to identify post-receptor events important in mediating IL 2 function. [Ca++] mobilization, protein kinase C activation and phosphoinositol hydrolysis have all been found to correlate with IL 2 induction of genes such as the IL 2 receptor, gamma interferon and the proto-oncogenes, c-fos, c-myb and c-myc. The IL 2 stimulation of IL 2 receptor transcription results in an increase of low affinity IL 2 receptors on the cell surface. The IL 2 receptor was shown to be present on immature thymocytes, monocytes, mast cells and hematopoietic progenitor cells, suggesting that the IL 2 receptor plays a important role in growth control of hematopoietic growth factors other than T-cells. Also, studies were conducted on the mechanism by which myeloid leukemic cells differentiate. Murine myelomonocytic leukemia cell lines variants studied included, WEHI-3B D+ which can be induced to differentiate into monocytes and WEHI-3BD-, which is unresponsive to these inducers (PMA and G-CSF). The unresponsive cell line was induced to differentiate to granulocytes and monocytes by 1,25 dihydroxycholcalciferol (1,25 (OH)2 D3), the biologically active metabolite of vitamin D3, suggesting that leukemic cells unresponsive to agents which act at the cell surface retain their ability to differentiate in response to agents that bypass the plasma membrane. The role of transforming growth factor B (TGF-B) on hematopoiesis was also investigated. Studies using the most immature murine hematopoietic cells that respond to IL 3 showed that the multipotent cell development but not unipotent cell development was inhibited by TGF-B. Thus, the ability of TGF to block hematopoietic progenitor cell growth and differentiation depends on the differentiated state of the cell and suggests that TGF may be an important regulator of hematopoietic cell growth.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Francis W. Ruscetti	Senior Investigator	LS LMI NCI
Maria C. Sparks	Guest Researcher	LS LMI NCI
Garwin K. Sing	Visiting Fellow	LS LMI NCI

MAJOR FINDINGS

I. Protein Kinase C Translocation, [Ca++] Mobilization, and Phosphoinositol Hyrolysis Correlates with Activation of T-cell Specific Genes

Regulation of the expression of the Tac antigen, a component of the high affinity IL-2 receptor was studied under the influence of various stimuli such as IL 2, an antigen-receptor agonist (anti-T3), phorbol esters and phytohemagglutinin (PHA). Phorbol esters stimulated de novo acquisition of Tac antigen, which was associated with the subcellular redistribution of protein kinase C (PK-C) from cytosol to particulate membranes of human T lymphocytes. PHA and anti-T3 (alpha-T3) antibody also stimulated a transient redistribution and activation of PK-C which reached a maximum within 20 minutes after stimulation. Both phorbol esters and alpha-T3 could increase Tac expression and stimulate PK-C translocation on 5 day and 12 day activated T cells, which were at the Go/G1 stage of the cell cycle due to IL 2 deprivation. Tac antigen specific mRNA was seen in the nucleus within 2 hours after stimulation. In contrast, IL 2 alone could only increase Tac expression and stimulate PK-C translocation on 5 day but not day 12 activated T cells. IL 2 synergizes with alpha-T3 and phorbol ester for the regulation of Tac expression. Although IL 2 increased expression of Tac, the majority if not all of these receptors possessed low affinity for IL 2. These data suggest that the activation of PK-C is a common transmembrane signal shared by IL 2 and antigen-stimulation. The results also imply that PK-C activation is needed for the regulation of Tac antigen expression. Similiar results were observed studying [Ca++] mobilization and phosphoinositol hydrolysis.

II. The Gene for the IL 2 Receptor is Expressed and the Gene Products are Present on the Cell Surface of a Variety of Non-lymphoid Cells

There are remarkable similarities in the intracellular and genetic events that occur when lymphoid and hematopoietic cells are exposed to their specific growth factors. The interleukin 2 (IL 2) receptor, whose cell surface expression is an absolute requirement for the growth and differentiation of lymphoid cells, was detected on various non-lymphoid hematopoietic cell types in this study. Cell lines consisting either of granulocyte-macrophage precursors or mast cells, which are dependent on interleukin 3 (IL 3) for their growth expressed high levels of the IL 2 receptor on their surface. Analysis of the binding characteristics of these receptors with 125I labeled recombinant IL 2 revealed that only receptors with low affinity for IL 2 were present on these cells. Addition of purified recombinant IL 3 to these cell lines led to an increase in IL 2

receptor gene expression within one hour in isolated nuclei. This IL 3-induced increase was also manifested with an increase in the number of IL-2 receptors on the cell surface which was maximal within 24 hours. Addition of 10,000 units of IL 2 to these cells had no apparent effect on their growth or differentiation. Whether the presence of the receptor possessing only low affinity for IL 2 on hematopoietic cells means that this receptor is involved in some important metabolic event in hematopoiesis remains to be maintained. In a similar manner, IL 2 receptors with low affinity for IL 2 have been found on the most detectable immature thymocytes, activated monocytes/macrophages and activated-natural killer cells.

III. Myeloid Leukemic Cells Unresponsive to Normal Cell Surface Signals can be Differentiated with Vitamin D3

The murine myelomonocytic leukemia cell line WEHI-3B D+ which differentiates in response to granulocyte colony stimulating factor (G-CSF) can also be induced to differentiate into monocyte-macrophages by phorbol myristate acetate (PMA) treatment. In contrast, the WEHI-3B D- subline which is unresponsive to G-CSF and PMA can be induced to differentiate to granulocytes as well as monocytes by 1,25 1,25 dihydroxycholcalciferol (1,25 (OH)2 D3), the biologically active metabolite of Vitamin D3. A newly developed variant of the WEHI-3BD-line, named WEHI-3B D+G, which was responsive to G-CSF, but not to PMA, could also be differentiated to granulocytes by 1,25 (OH)2 D3. Although vitamin D3 has been reported to induce macrophage differentiation in responsive tumor cells, this is the first demonstration that 1,25 (OH)2 D3 can induce granulocyte differentiation. In both differentiation pathways, cessation of cellular proliferation accompanies changes in morphologic and cytochemical properties of the cells. This suggests that leukemic cell lines unresponsive to differentiation agents acting at the cell surface retain their ability to differentiate in response to agents that do not act via the plasma membrane such as 1,25 (OH)2 D3, which has cytosolic/nuclear receptors. Vitamin D3 could act through different cellular pathways inducing differentiation or by bypassing only the first step of a common differentiation cascade used by agents with cell surface receptors such as CSF. These results suggest that low doses of 1,25 (OH)2 D3 may be useful in combination with hematopoietic growth factors (CSFs) as therapeutic agents to induce leukemic cell differentiation in vivo.

IV. Expression of the Nuclear Proto-oncogenes (c-fos, c-myb and c-myc) is not Sufficient to Induce Hematopoietic Cell Growth or Differentiation

Transcriptional activation of the cellular proto oncogenes (c-onc), by interleukin 2 (IL 2) and interleukin 3 (IL 3) was studied in responsive cell lines by nuclear run off transcription assays. Both recombinant human IL 2 which stimulates the murine T lymphocyte clone CT6, and recombinant Cos-7-derived IL 3 which stimulates the murine myeloid FDC-P1 line both induced a sequential increase in c-fos, c-myc, and c-myb mRNA synthesis. It has been also shown that both IL 2 and 3 stimulate the activation of protein kinase C (PK-C) in their respective interleukin dependent cell lines. PMA, a direct activator of PK-C, also resulted in a similar temporal induction in mRNA synthesis for the same c-onc genes. An increase in the expression of the c-fos, c-myc, and c-myb mRNAs was confirmed by Northern blot analysis. These results indicate that PMA and lymphokine growth factors regulate early c-onc gene transcription by similar

biochemical pathways. Since both phorbol ester and interleukins activate PK-C, this phosphotransferase system appears to be an important pathway in gene activation by these agents. The data suggest that these structurally unrelated growth factors promote growth in cells of distinct histologic origins by similar mechanisms involving PK-C activation. Transcription of c-fos, c-myc, and c-myb proto-oncogenes is rapidly increased by interleukin 2 and interleukin 3 in growth factor-dependent lymphoid and myeloid cell lines. Phorbol esters, direct activators of protein kinase C, also stimulate transcription of these proto-oncogenes but not proliferation in these cells, suggesting that the common biochemical events shared by PMA and interleukins do not alone account for the mitogenic effects.

The murine myelomonocytic leukemic cell line Wehi-3BD+ can differentiate into monocytes-macrophages in response to granulocyte colony stimulating factor (G-CSF) and phorbol myristate acetate (PMA). A variant which cannot be induced to differentiate with these agents, can differentiate after treatment with 1,25 dihidroxycholecalciferol, the biological active metabolite of vitamin D3, can be induced to differentiate mainly into granulocytes. The role of the proto-oncogenes myc, myb and fos, encoding for nuclear proteins was evaluated during the induction of myeloid cell differentiation. C-myc, c-myb, and c-fos mRNA levels were measured in both phenotype Wehi3B cell lines following treatment with PMA and vitamin D3. Utilizing the myeloid leukemic phenotypic variants: the Wehi-3BD- and Wehi-3BD- line, we questioned whether the ability or lack of ability of those cell lines to differentiate in response to PMA and vitamin D3 was correlated with the modulation of these cellular proto-oncogenes and whether modulation of the expression of those genes correlated with induced terminal differentiation of either monocyte or granulocyte phenotype. Our results showed that although PMA did not induce phenotypic differentiation of the Wehi-3BD- cells, modulation of proto-oncogenes was seen. PMA induced a reduced expression of c-myc and c-myb and stimulated significant expression of c-fos. On the other hand, vitamin D3, which did induce phenotypic differentiation of the Wehi-3BD- cells, also induced a reduced expression of c-myc and c-myb, but in contrast to PMA only induced moderate increased expression of c-fos in those cells. Wehi-3BD- cells which differentiate into macrophages in response to PMA, but not vitamin D3, only showed small changes in c-myc, c-myb, and c-fos expression after PMA or vitamin D3 treatment. Thus, modulation of expression of these genes does not correlate with the ability of the cells to be induced to differentiate.

V. Different Mechanisms of Proto-oncogene Induction in Hematopoietic Cells: Interleukins, Phorbol and cAMP

Transcriptional activation of the cellular proto-oncogenes c-fos and c-myc, in response to phorbol myristic acetate (PMA) and IL 2 was studied in a responsive cell line by nuclear run-off transcription assay. Recombinant human IL 2, which stimulates the murine T lymphocyte clone CT6, induced a sequential increase in c-fos and c-myc. It has also been shown that IL 2 stimulates the activation of PK-C in the CT 6 cell line. PMA, a direct activator of PK-C, also resulted in a similar temporal induction in mRNA synthesis for the same cellular proto-oncogenes. An increase in the expression of c-fos and c-myc mRNAs was confirmed by Northern blot analysis. However, PMA stimulated higher steady state levels of c-fos than c-myc mRNA, while IL 2 stimulated higher levels of c-myc than c-fos

mRNA. We investigated whether PMA and IL 2 augmented cellular oncogene transcription by distinct pathways. Because cAMP has been reported to stimulate cell proliferation, we have studied the possible induction by PMA and IL 2 of cAMP levels in this cell line. PMA increases cAMP levels in this cell line while IL 2 does not. The increase in cAMP by PMA correlates with changes in transcription and steady state levels of proto-oncogene mRNAs. Similar results were obtained with dibutyryl cAMP and with PDD, a phorbol ester which does not activate PK-C. At the transcriptional level, both dibutyryl cAMP and PDD activated the same pattern of cellular proto-oncogenes.

Therefore, our results suggest the existence of at least two post-receptor pathways by which c-fos and c-myc mRNAs can be induced in the CT6 cell line. One pathway uses protein kinase C activation by IL 2 via phosphatidyl inositol turnover. A second pathway uses cAMP which, in eukaryotes, is thought to activate cAMP dependent protein kinase. This pathway appears also to be affected by PMA. The PMA induction of cAMP levels appears to be specific for this CT 6 cell line.

VI. Differential Effect of Protein Synthesis Inhibition on Proto-oncogene Transcription in Lymphoid Cells

The mechanisms by which growth factors promote cellular proliferation is not clearly understood. We have found that treatment of lymphoid cells with growth promoting factors such as IL 2 and IL 3 leads to the transient expression of the cellular proto-oncogenes c-fos and c-myc.

In both IL 2 and IL 3 dependent cell lines, the kinetics of activation and repression of transcription is distinct for c-fos and c-myc. C-fos transcription peaks at 30 min after growth stimulation, whereas c-myc expression is maximal approximately 60 to 120 min after interleukin treatment. The observation that c-fos gene expression is stimulated before the increase in transcription of c-myc raises the question of whether activation of the synthesis of c-fos or other rapidly induced proteins might be required for subsequent activation of genes such as myc.

We have used cycloheximide, a protein synthesis inhibitor, to investigate whether synthesis of new proteins plays a role in the rapid induction and subsequent repression of the transcription of these genes. Nuclear run off transcription assays of CT6 indicated that when added before growth factor stimulation, cycloheximide potentiates the transcription of c-fos. In contrast cycloheximide completely inhibited the increase transcription of c-myc. Nevertheless, the pretreatment with cycloheximide caused a superinduction of the steady state mRNA levels of both proto-oncogenes.

These results suggest that: 1) in CT 6 c-fos transcription is activated by a protein synthesis independent mechanism, while c-myc stimulation requires new protein synthesis; and 2) the effect of cycloheximide at the steady state level appears to be affecting the stability of the mRNA.

VII. Regulation of Normal Hematopoiesis by TGF-B

The effect of TGF-B on factor-induced murine bone marrow (BM) proliferation and colony formation was studied. TGF-B inhibited IL 3-induced 3H-thymidine incorporation of freshly aspirated BM cells in a dose dependent manner with an ED-50 of 0.05 ng/ml (2 pM). The same result was obtained in the presence of low doses of IL 3 (5 u/ml) or saturating amounts of IL 3 (50 u/ml). In contrast, TGF-B showed no effects on G-CSF or GM-CSF driven proliferation over a wide range of TGF-B concentrations. Using soft agar in vitro assays, TGF-B suppressed IL 3-promoted CFU-GMs at 0.25 ng/ml (10 pM), the concentration which inhibited maximal 3H-thymidine incorporation. In agreement with proliferation data, TGF-B had no inhibitory effect on G-CSF or GM-CSF promoted colony formation. The data suggest that TGF-B exerts a differential effect on factor induced colony formation.

To determine whether TGF-B was exerting its effects on the early stages of hematopoiesis, erythropoeisis was examined. First, the effects of TGF-B on the late stages of erythropoeisis demonstrated that erythropoeitin (epo) induced proliferation and colony formation was uneffected. Second, IL 3-promoted proliferation and differentiation of CFU-GEMM colonies in soft agar, representing the earliest hematopoietic colonies which can give rise to red cells in the presence of epo, were inhibited in the presence of TGF-B. Only clusters (< 20 cells) of differentiated granulocytes and macrophages were observed.

The data suggest that TGF-B may be acting on early progenitor populations, therefore, the effect of TGF-B on a variety of IL 3 dependent cell lines representing myeloid progenitors blocked in differentiation was examined. IL 3-induced proliferation of NFS-60 was inhibited by TGF-B in a dose dependent manner with an ED-50 of 4-6 pM. In addition, since NFS-60 can respond to a number of growth factors, these were also examined. TGF-B inhibits GM-CSF and G-CSF induced proliferation with ED-50s pf 4-6 pM, and CSF 1 and IL 4 induced proliferation at 4-6 pM concentrations of TGF-B. This effect was consistently observed among IL 3 dependent cell lines. This data suggest that TGF-B can suppress the proliferation of early hematopoietic progenitors independent of the hematopoietic growth factor used.

To determine whether TGF-B could inhibit the proliferation and differentiation of IL 3-induced THY-1+ cells which represent early hematopoietic progenitors, THY-1+ populations were sorted and grown in soft agar assays. The results demonstrated that 1) the THY-1+ cells contain CFU-GEMM-erythroid colonies in the presence of epo; 2) that TGF-B inhibits the colony formation of CFU-GEMM while permitting the growth of monolineage colonies of G- or M-, and a 50-60 percent reduction in total colony number.

Evidence thus far also indicates that CSF 1 derived BM macrophages can respond to IL 3 or GM-CSF and that these mature committed end stage cells are not inhibited by TGF-B.

VIII. The Regulation of Hematopoietic Tumor Cells by Transforming Growth Factor B

Transforming growth factor (TGF) is present in a variety of tissues, and has both growth promoting and inhibiting properties. It has been suggested that

this class of growth factors may perform a more generalized function in such processes as embryogenesis, wound healing, bone resorption and tissue stem cell proliferation. Moreover, TGF acts as an important immunomodulatory protein for cells of the immune system, inhibiting proliferation and Ig secretion of stimulated B cells as well as inhibiting IL 2 dependent T cell proliferation. Recently, it has been realized that bone is a rich source of TGF, and that this growth factor is abundant in demineralized bone matrix, BM and fetal liver, these being the primary active sites for hematopoiesis. This work was therefore undertaken to investigate the effects of TGF on the growth of normal and leukemic granulocyte-macrophage (CFU-gm), erythroid (CFUe, BFUe) and multipotential (CFU-GEMM) progenitor cells. In normal human BM, TGF caused a dose-dependent inhibition of colony formation, particularly of the early CFU-GEMM progenitors. On the other hand, G-CSF induced colony formation was not significantly inhibited by TGF, suggesting that cells in the later stages of the differentiation cycle are less susceptible to TGF inhibition. BM cell progenitors from patients with chronic myelogeneous leukemia (CML) were approximately 10x more sensitive to inhibition by TGF than from normal donors. Nevertheless, a small population of these cells remains insensitive to TGF-B inhibition, since the degree of proliferation at maximum inhibition is still above that of background inhibition in the absence of colony stimulating factor. Although CML cells were more sensitive to TGF than normal cells as measured by uptake of 3H-thymidine, in the colony-forming assay, the reverse was true. Since the proliferation assay is measured after 72 hr of culture whereas the CFU-C assay is measured after 2 wks, it would suggest that inhibition of cell growth in CML cells is greater, although less prolonged as compared to normal cells. Unlike normal BM, CML cells were also sensitive to TGF inhibition in the presence of G-CSF. To study the effects of TGF on BM cell differentiation, a number of cell lines of different lineages were tested for sensitivity to TGF. Cell lines of both myeloid-monocytic and erthyroid lineages were sensitive to TGF inhibition, although there were also lines such as HL-60 and K562 which were insensitive. Although TGF inhibits IL 2 driven proliferation of normal T lymphocytes, it was not inhibitory for the growth of any of the transformed T cell lines tested.

Recently, two forms of TGF have been described, namely TGF-1 and TGF-2. Both share very similar but not identical amino acid sequences and share common receptors. They are also equally effective in inducing chondrogenesis in vitro as well as inhibiting IL 1 mediated proliferation of rat thymocytes. In contrast, we have found a differential degree of inhibition with TGF-1 generally displays a 10-fold greater degree of inhibition than TGF-2. In the CFU-C assay, TGF-1-treated cells showed a dose-dependent inhibition of colony-forming cells of all lineages, whereas no inhibition could be seen with TGF-2, suggesting that its inhibitory effect, as detected in the proliferation assay, is of a transient nature. No synergy was seen between TGF-1 and 2 together since the 50% effective dose of both factors added together was equal to that of TGF-1. However, the degree of proliferation in cells treated with TGF-1 and 2 in the picomolar range remained well below that of the controls, as opposed to TGF-1 alone, which at this concentration, failed to inhibit progenitor cell proliferation. It therefore appears that TGF-1 and 2 have different roles to play in the control of hematopoiesis.

The ability of other cytokines, known to affect the growth of BM haematopoietic progenitor cells to synergise with TGF was also tested. First, tumor necrosis factor (TNFa) which has a number of different properties, including the inhibition of progenitor cell proliferation, was tested. It has also been reputed to have antiviral effects, although it was not found to inhibit the growth of HTLV I, II, and HIV-infected cell lines using concentrations ranging from 1000 to 1 unit of TNF, nor did it inhibit the growth of normal lymphoid cells such as BCGF-stimulated B cells. No synergy was seen between TGF and TNF since the 50% effective dose on the proliferation assay was similar using TNF and TGF or TNF alone, lying in the range of 10 u/ml. However, in the CFU-C assay, using a constant amount of TGF at 0.1 ng/ml, colony formation was completely inhibited all concentrations of of TNF tested, ranging from 100 units to 1 u/ml, suggesting a possible therapeutic use for these 2 regulatory factors acting in concert. A similar co-operative effect was seen between IFNa and TGF, where IFNa alone only inhibited colony formation at concentrations of 100 u/ml, whereas in the presence of 0.1 ng/ml TGF, complete inhibition of colony formation was seen at 10 and 1 u/ml concentrations of IFNa.

IX. Effect of v-abl on Hematopoietic Development

The myelogenous leukemic cell line NFS-60 is an hematopoietic progenitor blocked in differentiation. Its growth factor requirements can be met by a number of hematopoietic growth factors. Another cell line, 32D CL-23, required IL 3 for its growth. These two cell lines were infected with Abelson murine leukemia virus (A-MuLV) containing the onc gene v-abl. Subsequent selection of A-MuLV-infected NFS-60 and 32D CL-23 in growth factor free medium resulted in the establishment of growth factor independent clones (NFS-60-ABL's, 32-DCL-ABL's). The abrogation of factor requirement by v-abl has been demonstrated in other cells. These clones expressed abl specific message by Northern analysis as well as the p120 gag-abl fusion protein. Although v-abl led to the abrogation of factor dependence in both cell lines, marked differences were observed in the ability of these two lines to undergo differentiation. Specifically, NFS-60-ABL's spontaneously differentiated into morphologically identifiable macrophages at various stages in differentiation with cells containing irregularly shaped nuclei and lightly stained vacuolated cytoplasm, and a high cytoplasm to nuclear ratio. In comparison, the parental NFS-60 contained immature myeloblasts with scant cytoplasm and oval nuclei. The introduction of v-abl into NFS-60 has overcome the block in differentiation in addition to abrogating factor dependence. In contrast, 32D CL-23-ABL, does not undergo spontaneous differentiation when it loses its dependence on the factor. These observations suggest that in addition to the abrogation of factor dependence, expression of v-abl at certain stages of hematopoietic development promotes differentiation down the monocytic lineage. In addition to the these results, NFS-60-abl exhibit subsequent non-specific esterase staining (macrophages), become positive for MAC-1 (macrophages), and show decreased levels of THY-1 expression-associated with differentiation, as well as increased levels of Fc receptor expression based on facs analysis.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09228-07 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Further Characterization of Natural Killer (NK) Cells in the Rat

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

PI:	C. W. Reynolds	Head, Cellular and Molecular Immunology Section	LEI, NCI
Others:	D. Reichardt	Biologist	LEI, NCI
	M. Galli	Visiting Fellow	LEI, NCI

COOPERATING UNITS *(if any)*

Pittsburgh Cancer Institute, Pittsburgh, PA (J. Hiserodt); University of Oklahoma (J.A. Murphy); Immunology Branch, Division of Cancer Biology Diagnosis, National Cancer Institute (P. Henkart); Harvard Medical School (R. Stevens).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The present studies further characterize natural killer (NK) cells in the rat. The results demonstrate substantial differences in the ability to recirculate between the cells which mediate NK activity or large granular lymphocytes (LGL) and normal rat T cells. Studies on lymphokine-activated killer (LAK) cells indicate that the major precursor of LAK activity in the rat is an LGL. Studies with normal rat LGL and the LGL tumor lines have demonstrated a lack of T cell receptor and interleukin 2 (IL2)-receptor gene expression, even in LGL tumor lines which respond to IL2 stimulation. Analysis of the granules from LGL have led to the isolation of a cytolytic protein (cytolysin), at least two BLT-esterases, LGL specific chondroitin sulfate A glycosaminoglycans and proteoglycans, and an anti-fungal molecule which inhibits the growth of Cryptococci neoformans. These differences between the LGL and T cells in a number of basic biological properties suggest that LGL represent an entirely separate, third lineage of lymphocytes, distinct from both T and B cells.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Craig Reynolds	Head	CMIS	LEI	NCI
Della Reichardt	Biologist	CMIS	LEI	NCI
Maria C. Galli	Visiting Fellow	CMIS	LEI	NCI

OBJECTIVES

The objectives of this project are: (1) to investigate a number of general biological properties of large granular lymphocytes (LGL), (2) to study the role of LGL in the generation of lymphokine-activated killer (LAK) cells, (3) to further characterize the transplantable spontaneous LGL leukemias in F344 rats, (4) to analyze specific cell surface antigens and membrane receptors responsible for target cell lysis, and (5) to examine the mechanism(s) by which LGL mediate their functional activity.

MAJOR FINDINGS

I. Characteristics of LGL

Detailed studies on isolated rat LGL have demonstrated these cells to be a distinct population of cells, about 75 μm2 in size, positive for acid phosphatase and β-glucuronidase; and negative for alkaline phosphatase, esterase, peroxidase and lysozyme. Rat LGL are an antigenically distinct population of cells that share a number of cell surface antigens with monocytes, suppressor/cytotoxic T cells and polymorphonuclear leukocytes (PMN).
Our recent studies on the biology of LGL have demonstrated that unlike T cells, LGL are nonrecirculating lymphocytes with a very rapid precursor turnover time (approximately 10 days). These results are consistent with our observations of LGL present in the red pulp of the spleen but absent from the areas of lymphocyte recirculation; spleen (white pulp), lymph nodes and thoracic duct. Analysis of the interleukin 2 (IL2)-receptor using monoclonal antibodies and Northern blot analysis for mRNA has also demonstrated the absence of a classical 70 kD chain for the IL2-receptor. This result is in spite of the fact that LGL respond to IL2 by 1) increasing their NK activity, 2) growth, 3) activation of LAK activity and 4) production of interferon-γ (IFN-γ) and interleukin 1 (IL1). Further studies on the nature of the IL2-receptor in LGL are currently underway.

II. Role of LGL in the Generation of LAK Cells

In collaboration with Dr. John Hiserodt (Pittsburgh Cancer Institute), these studies demonstrated that LAK activity could be generated from spleen, peripheral blood, lymph nodes, thymus and bone marrow. Spleen cells and peripheral blood lymphocytes respond quickly to recombinant IL2 (rIL2) giving peak LAK activity by 3 to 5 days in culture. Thymus and bone marrow cells were slower to respond peaking by 7 to 9 days in culture. We interpret these data to indicate that thymus and bone marrow contain low levels of LAK progenitor cells which must first expand prior to developing LAK activity. Cells obtained from peritoneal exudate or neonatal spleen cells were unable to generate LAK activity due to active suppressor mechanisms.

Extensive surface marker analyses was performed to determine the phenotype of the LAK precursor and effector cells. LAK precursors expressed surface markers characteristic of NK/LGL since the generation of LAK activity could be significantly reduced by treatment of spleen cells with antibodies to LGL determinants (asialo GM1 or laminin) but not with antibodies to the pan T cell marker, R1-3B3. Furthermore, depletion of granular lymphocytes by the lysosomotropic agent L-LME depleted NK activity and prevented the generation of LAK activity. This same treatment, however, did not affect T cell proliferative responses to Con A.

Preparations of ultra-pure LGL (specifically devoid of T cell contamination by panning with the R1-3B3 MoAb) were fully capable of generating LAK activity. These same populations of ultra-pure LGL demonstrated strong proliferative responses to rIL2 alone. Similar preparations of highly purified resting T cells were unable to generate efficient LAK activity, even when cultivated with an additional activating signal, Con A.

Cell sorting analysis of the LAK effectors demonstrated that LAK effectors clearly sorted into the pan T (R1-3B3) and helper T (CD4) negative populations. The majority (>90%) of the LAK activity sorted into the CD8+, Ia+, and laminin+ populations. These studies indicate LAK cells can be generated from a variety of lymphoid organs in rats and indicate that the major (if not only) cells responding to rIL2 to generate LAK activity are LGL/NK cells.

III. Comparison of Spontaneous Rat LGL Leukemias With Normal Rat LGL

Our recent discovery of spontaneously occurring LGL tumors in aged Fischer (F344) rats has provided a very useful system for obtaining a large number of LGL for detailed biochemical and genetic studies. The rat NK (RNK)-leukemias are morphologically identifiable as LGL with azurophilic granules in the cytoplasm. The RNK lines efficiently kill the NK-sensitive targets (YAC-1, G1-TC), but have little or no activity against the NK-resistant targets [(C58NT)D and P815], and have been used as a convenient source of highly active cells for the isolation and analysis of recognition receptors, cytoplasmic granules, and lytic machinery. We have recently described three new rat LGL tumor lines which can be cultured in vitro without the loss of LGL morphology or function. Two of the lines were initiated in IL2 with the subsequent establishment of both IL2-dependent and IL2-independent sublines. In spite of the IL2 dependence of some lines for growth, no rat IL2 receptor expression was observed using a monoclonal anti-receptor antibody (OX-39) or Northern blot analysis of mRNA using an IL2-receptor specific probe. The morphology of all the cultured LGL tumor lines was consistent with that of LGL; with a kidney-shaped nucleus and prominent azurophilic granules in the cytoplasm. Like normal rat LGL the lines were CD2 (OX-34), CD8 (OX-8), CD45 (T200) and asialo GM1 positive; but negative for the pan-T cell CD5 (R1-3B3, OX-19) marker, CD4 (W3/25) and a variety of other T cell and macrophage associated antigens. Unlike most normal rat LGL, the LGL tumor lines were strongly Ia (OX-6) positive. Two of the three lines killed a variety of NK-sensitive tumor targets but not NK-resistant target cells. All the cultured LGL tumor lines exhibited significant antibody-dependent cellular cytotoxicity (ADCC). In spite of the addition of exogenous IL2 or even the continuous presence of high concentrations of IL2, no LAK activity was observed in any of the in vitro grown

lines. All three lines were shown to lack mRNA for either the α or β chains of the T cell receptor. The existence of these lines with high levels of NK activity in the absence of LAK activity now provides a mechanism to study a number of in vitro questions requiring very large numbers of functionally active "pure" LGL in the absence of any contaminating cells.

IV. Expression of T Cell Receptor Molecules on LGL

Along these lines, we have recently examined highly purified, freshly isolated LGL from both the human and the rat, as well as the LGL tumors for the expression of the α, β, or γ-chains of the T cell receptor for antigen. The results demonstrate that all these LGL preparations express no RNA indicative of a functional transcript for the α, β, or γ-chains. In addition, Southern blot analysis of rat LGL tumor DNA demonstrated no β or γ-chain rearrangement. Taken together, these results strongly suggest that a functional T cell receptor is absent from LGL, thus making it extremely unlikely that this molecule is involved in antigen recognition by LGL. Studies are currently underway to identify the nature of this antigen receptor on LGL.

V. Analysis of Functional Molecule(s) from the Cytoplasmic Granules of LGL Tumor Lines

The LGL leukemias have previously been shown to be an excellent source of cells for projects which involve the isolation and characterization of subcellular products which may be present in LGL in very small amounts. The use of LGL tumors for these studies is critical since the amount of material required to do these detailed experiments clearly cannot be obtained from normal LGL.

In collaboration with Dr. Pierre Henkart (Z01 CB 05018-17), we have identified at least one molecule from LGL granules which is highly cytolytic. This molecule has not been found in the cytoplasmic granules from other cells; including resting T cells, PMN's, mast cells and macrophages. Antibodies produced in rabbits against the LGL granules were found to be highly inhibitory for both rat and human NK and ADCC activity. Further purification of the cytolytic molecule within the granules suggests that it is a protein of approximately 60,000 MW and acts via polymerization and insertion into the target cell membrane (similar to poly C9 complement pores).

In addition to this cytolytic molecule we have been able to identify at least two BLT-esterase molecules (MW 60 kD and 25 kD) in the granules of LGL. Each esterase can be separately isolated and antisera against each esterase specifically inhibits their respective activity. Studies are currently underway to examine their possible functions in vivo and in vitro.

In collaboration with Dr. Richard Stevens (Harvard Medical School) we have also examined rat LGL for the presence of proteoglycans and glycosaminoglycans in their cytolytic secretory granules. When isolated rat LGL tumor cells were incubated with [35-S]sulfate and the 35-S-labeled macromolecules were purified by density-gradient centrifugation, they eluted on Sepharose CL-4B columns predominantly as approximately 500 kD macromolecules. Pulse-chase experiments revealed that these larger macromolecules were proteoglycans that, with time, were processed to glycosaminoglycan-sized macromolecules (approximately 85 kD).

As assessed by their susceptibility to chemical and enzymatic degradation and by high-performance liquid chromatography, the LGL-associated proteoglycans bore almost exclusively chondroitin sulfate A glycosaminoglycans. Northern blot analysis, using a gene-specific probe revealed that both normal peripheral blood and transformed rat LGL expressed the same 1.3 kb mRNA that encodes the peptide core of the proteoglycan present in the secretory granules of rat and mouse mast cells. In vivo radiolabeling of rat LGL tumor cells and isolation of their intact granules established that these glycosaminoglycans compartmentalized with the cytolytic activity. These results suggest that the negatively-charged macromolecules may play a role in the regulation, packaging and possible delivery of the cytolysins and basically-charged serine proteases discussed previously.

The granules have been shown to affect the in vitro growth of the fungus Cryptococci neoformans (Dr. June Ann Murphy, University of Oklahoma). Pretreatment of Cryptococci with whole granules or soluble granule contents completely inhibited the growth of this microorganism. Addition of antigranule Ab or removal of Ca+2/Mg+2 blocked this effect, suggesting that a similar molecule may be affecting the growth of these fungus and causing the lysis of tumor cells. The LGL granules from these tumors have also been shown by Dr. Arnold Greenberg (University of Manatoba) to contain molecule(s) which: 1) will activate the bacteriocidal activity of macrophages and 2) leads to the chemotaxis of monocytes and LGL. Further characterization of the granule associated molecules and a comparison with the antitumor and antifungal activity of LGL granules is underway.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09247-07 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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:	C. W. Reynolds	Head, Cellular and Molecular Immunology Section	LEI, NCI
	D. Reichardt	Microbiologist	LEI, NCI
	T. Bino	Visiting Fellow	LEI, NCI

COOPERATING UNITS *(if any)*

Immunology Branch, Division of Cancer Biology Diagnosis, National Cancer Institute (P. Henkart); Pittsburgh Cancer Institute (R. Herberman), Program Resources, Inc. (J. Rossio and R. Winkler-Pickett)

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

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

Natural killer (NK) cells and K cells mediating antibody-dependent cellular cytotoxicity have been shown to be large granular lymphocytes (LGL). The majority of LGL form lytic conjugates with a wide variety of NK-susceptible target cells. The relationship of soluble factors to this cell mediated lysis, has been examined using NK cytotoxic factors (NKCFs) as the mechanism of action. Three distinct steps have been defined for NKCF: a) production, b) binding to targets, and c) subsequent target lysis. With procedures able to independently measure these events, a variety of agents which have been reported to inhibit NK cell-mediated killing are being tested to determine their site of action. These NKCFs are produced by LGL and have a general specificity pattern similar to intact killer cells. Comparisons were made between NKCF and recombinant lymphotoxin (LT), tumor necrosis factor (TNF), and leukoregulin. The results demonstrated that NKCF is distinct from both these cloned factors. We have now used rat LGL cell lines as a source for NKCF and begun its molecular cloning with the aid of the specific monoclonal antibody to NKCF. In addition to lytic mechanisms, studies are proceeding to define the receptors and structures involved in NK recognition. In studies of NK target antigens on K562 cells, a MoAb was developed which was capable of blocking LGL binding and lysis. An anti-idiotypic antibody (anti-ID) has also been developed, anticipating that this antibody would recognize the NK receptor and aid in its identification. This anti-ID antibody is reactive with a 85 kD protein and block LGL binding and target cell lysis. Finally, when LGL were pretreated for 18 hrs with the F(ab')2 anti-ID antibody, there was significant enhancement of lysis and production of IFN-γ. It will be important in future studies to characterize the expression of the 85 kD molecule on activated cells that have reported NK activity. Further biochemical characterization of the NK-R and its cloning are anticipated in future studies.

PROJECT DESCRIPTION

PERSONNEL

John Ortaldo	Chief		LEI	NCI
Craig W. Reynolds	Head	CMIS	LEI	NCI
Della Reichardt	Microbiologist	CMIS	LEI	NCI
Tamar Bino	Visiting Fellow	CMIS	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 the nature and mechanism of cytotoxicity by LGL and to attempt to isolate and characterize soluble cytotoxic factors;

3. To study, isolate and characterize the nature of NK effector cell receptors;

4. To study, isolate and characterize the nature of NK target structures.

MAJOR FINDINGS

I. NK Recognition

The nature of the structure(s) on target cells that are recognized by NK cells is presently not well understood. Studies involving indirect methods to examine the specificity of human and mouse NK cells have led to the conclusion that reactivity is quite broad but demonstrates a selective pattern of killing. Liposome studies using target moieties prepared from K562 associated with but not from RL-male-1 (insusceptible to human NK) inhibited the binding of LGL to intact targets. In addition, the preparations obtained from K562 membranes also inhibited LGL binding to other human targets (Molt4, ALAB, Daudi) but failed to compete for binding of rat LGL with YAC (a rat NK target). However, membranes from RL-male-1, which failed to compete for binding with human LGL, demonstrated inhibition of rat LGL binding. In addition, neither membrane preparation inhibited ADCC binding to antibody-coated RL-male-1. The isolated, inhibitory membrane component appeared to be glycoprotein in nature. In studies of NK target antigens on K562 cells, a MoAb was developed which was capable of blocking LGL binding and lysis. These IgM monoclonal antibodies could remove target glycoproteins capable of inhibiting LGL binding to NK susceptible targets. Biochemical studies indicated that the major monoclonal binding was to carbohydrate determinants on the glycoprotein. Therefore, an anti-idiotypic antibody (anti-ID) has also been developed, anticipating that this antibody would recognize the NK receptor and aid in its identification. This anti-ID antibody is reactive with a 85 kD protein and block LGL binding and target cell lysis. Consistent with the idea that this antibody recognizes the NK receptor, F(ab')2 fragments of the anti-ID antibody were found to react only with CD3- LGL among the leukocytes tested. When LGL were pretreated for 18 hrs with the

F(ab')2 anti-ID antibody, there was significant enhancement of lysis as compared with normal rabbit serum (NRS) treated control. After pretreatment, levels of IFN-γ production were also increased. Identification of the NK-R will be important to future studies in distinguishing NK activity from activity of other cell types. The NK-R will be useful in clarifying NK cell lineage, and in the identification of the NK-R ligand. The defined NK-R can be used to identify NK active cells in mixed populations. Present studies are involved in studying the expression of the 85 kD molecule on activated cells that have reported NK activity (CD3+, Leu19+, T-cells, B cells, macrophages, etc.) to determine if these cells express and utilize the same receptor. MoAb to the 85 kD molecule are also being produced. Further biochemical characterization of the NK-R, and its cloning are anticipated in future studies.

II. Mechanism of Human NK Activity

Studies have been initiated with cytotoxic factors produced by NK cells (NKCFs). These factors have been shown to be produced in high quantity by NK cells after incubation with lectin or NK-susceptible targets. NKCF has a restricted pattern of lysis, similar to that of LGL. Using NKCF as a model for cytolysis by LGL, we have analyzed a variety of agents previously demonstrated to inhibit NK activity. All of the inhibitors will be tested for their effects on: 1) production of NKCF after target-effector interaction; 2) binding of NKCF to target cells; and 3) NKCF lysis (after 6 hrs of adsorption and washing) of targets. In addition to the above studies, purification studies were performed to begin biochemical characterization of human NKCF. The results indicated that radiolabeled NKCF has an apparent non-reduced molecular weight between 20,000 and 40,000. Culture supernatants from rat LGL tumors were shown as an excellent source of NKCF activity for NK-susceptible targets, MBL-2 and YAC-1. NKCF production from these rat tumor lines, unlike fresh lymphocytes, were constitutive. Mouse monoclonal antibodies were developed (A0287, A0462, and A0316) which significantly inhibit the NKCF cytolytic activity of these LGL-derived supernatants. These antibodies were shown to cross-react with human NKCF in a fashion similar to that seen in the rat. Of interest, these same monoclonal antibodies demonstrated no inhibition of L929 cytotoxicity from either LGL-derived supernatants or by recombinant murine or human TNF. To further examine the specificity of these antibodies they were chemically linked to Sepharose-4B and found to remove a significant proportion of the NKCF cytolytic activity from LGL supernatants, while not affecting the TNF reactivities in these preparations. These antibodies demonstrated significant inhibition of cell-mediated cytotoxicity by rat LGL against YAC-1 target cells. Biochemical analysis of labeled NKCF recognized by these anti-NKCF monoclonal antibodies (MoAbs) indicated the major reduced protein of 12,000 kD. The use of these MoAbs against NKCF should be very useful in further purification, biochemical characterization and molecular cloning of NKCF and in studying its role in a variety of cell-mediated cytotoxicity assays.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09256-05 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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:	A. Mason	Biologist	LEI, NCI
	H. Young	Expert	LMI, NCI
	F. Ruscetti	Head, Lymphokines Section	LMI, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., NCI-FRCF (J. Rossio, R. Overton, J. Talmadge; Medicine Branch, Division of Cancer Treatment (Dr. Ozols); Pittsburgh Cancer Institute (R. Herberman)

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

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

Natural, recombinant and hybrid recombinant alpha, beta, and gamma interferon (IFN) molecules have been shown to augment large granular lymphocytes (LGL), natural killer (NK), and killer (K) activity but vary widely in their potency relative to antiviral activity. Interleukin 2 (IL2) has demonstrated a potent ability to augment NK activity and the generation of killers against NK insensitive targets. This IL2-mediated augmentation appears to parallel production of IFN-γ by LGL, but abrogation of antiviral activity with anti-IFN-γ serum did not abolish NK boosting. Studies examined the regulation of LAK progenitors and effectors. Steroids (dexamethasone) consistently depressed lymphokine activated killer (LAK) progenitors. However, this agent had no effect on previously generated LAK effector cells. In addition, recombinant interleukin 1α (rIL1α) and β, IFN-α as well as G- and GM-CSF did not modulate LAK effectors or their progenitors. Numerous cytokines were examined (IL1α/β, IFNα, G- and GM-CSF) however, only IFN-γ was able to mediate a low but consistent enhancement of LAK progenitor activity. These in vitro results are consistent with the hypothesis that IL2 is the major inducer of LAK activity and that other recombinant cytokines do not appear to regulate either the progenitor or effector stages of LAK activity. In addition to NK activity, LGL have been shown to produce a variety of lymphokines (IL1, IFN, CSF, BCGF). A project investigating gene expression and regulation in highly purified human LGL and T cells is being conducted. Within 1 hr after IL2 treatment of freshly isolated human LGL, IFN-γ mRNA can be detected with IFN-γ protein in the culture medium within 4-6 hours of treatment. These results indicate that with certain stimuli LGL may be the predominant source of IFN-γ from peripheral blood lymphocytes. The methylation state of the T cell receptor β-chain gene (T-β) DNA of T cells, LGL, B cells and monocytes is being studied to determine if it can be used as a marker for different leukocyte populations.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John Ortaldo	Chief		LEI	NCI
Anna Mason	Microbiologist	CMIS	LEI	NCI
Howard Young	Expert	IS	LMI	NCI
Frank Ruscetti	Head	LS	LMI	NCI

OBJECTIVES

The objectives of this project are:

1. To study the factors regulating the activation and development of natural killer (NK) and related natural effector cells;

2. To analyze the interaction of natural effector cells with other components of the immune system;

3. To study cultures and clones of large granular lymphocytes (LGL) for their cytolytic activity and their production of soluble products in regulation of the immune system.

MAJOR FINDINGS

I. Regulation of Human NK Activity

In addition to interferon (IFN), interleukin 2 (IL2) potently augments human NK activity. Using highly purified LGL and recombinant IL2 (rIL2), the dose and kinetics of augmentation have been studied. Of considerable interest has been the ability of IL2 to augment NK activity in the absence of growth promotion and was not abrogated with monoclonal antibodies to TAC. This IL2 mediated augmentation results in the dramatic broadening of target cell selectivity and a striking augmentation in the degree of lysis observed. Studies were performed to examine the phenotypes of the progenitor and effector cells and compare these with the cells described lymphokine activated killer (LAK) cells. Both CD3+ CD16-, and CD3- CD16+ cells could mediate LAK lysis against fresh tumor cells. Using limiting dilution frequency analysis, it was demonstrated that a large percentage of the cytolytically active progenitors were present among the CD16+ NKH1+ CD3- progenitors. Accumulated data suggested that there was not a single unique progenitor for LAK but rather several subsets of lymphocytes capable of becoming cytotoxic in a nonsensitized fashion against a variety of fresh tumor cells. However, the NK population forms the largest single component of precursor cells for generation of LAK activity in the human peripheral blood. Studies have begun to examine the regulation of LAK progenitors and effectors by cytokines and steroids. Since the capacity to generate more potent LAK activity is potentially useful in clinical trials, there is considerable interest in determining whether other biological agents, besides IL2, are capable of regulating the ability to generate LAK activity. Steroids (dexamethasone) at doses of 1-1000 ng/ml consistently depressed LAK progenitors. However, this agent had no effect on previously generated LAK effector cells. This is of

considerable interest since some patients are receiving steroids prior to or during LAK therapy to minimize toxic effects of IL2. In addition, rILlα and β, IFN-α and -γ, as well as G- and GM-CSF were tested for their ability to modulate LAK effectors or their progenitors. None of these recombinant biologicals exhibited major synergistic or antagonistic activity in combination with IL2. IFN-γ was able to mediate a low but consistent level of enhancement in LAK activity when progenitor cells had been cultured with low doses (generally 1 U/ml) of this cytokine. Interestingly, high doses of IFN-γ (10,000 U/ml) significantly inhibited progenitors, presumably through their anti-proliferative effects. Therefore, these in vitro results are consistent with the hypothesis that IL2 alone induces LAK activity and that other recently described recombinant cytokines do not appear to regulate either the progenitor or effector stages of LAK activity.

II. Non-cytotoxic Function of Human CD3- LGL

In addition to their central role in mediating NK activity, there are indications that human LGL also have considerable immunoregulatory functions, including the ability to secrete a variety of cytokines. Highly purified populations of LGL, depleted of all detectable T cells and monocytes, could be stimulated to produce substantial levels of: 1) interferons, with the type depending on the stimulus; 2) IL2; 3) IL1; 4) colony stimulating factor; and 5) B cell growth factor. The secretion of different cytokines was dependent upon the stimulus used. These results have stimulated much interest in the NK cell as a potent immunoregulatory cell. In this regard, studies have been performed which demonstrate that IL2 (which is a potent activator of NK cytotoxicity as well as development of LAK-like activity in the LGL) is capable of stimulating transcription/translation of IFN-γ from purified populations of LGLs. Recombinant IL2 alone, using genetic probes with Northern blot analysis has been able to activate the message for IFN-γ from LGL but not T cells in as little as 6 hours. Present studies are designed to study these noncytolytic functions of LGLs and their relationship to other cells in the system, and are being extended to IL1, IL4, IL5 and other biologically relevant cytokines.

A project investigating gene expression and regulation in highly purified human LGL and T cells is being conducted with Dr. H. Young (Laboratory of Molecular Immunoregulation, BRMP). Within 1 hr after IL2 treatment of freshly isolated human LGL, IFN-γ mRNA can be detected with IFN-γ protein in the culture medium within 4-6 hours of treatment. CD3- CD16+ LGL require only a single signal for IFN-γ-production since phytohemagglutinin (PHA), phorbol myristate acetate (PMA), IL2 or ionomycin can each independently induce IFN-γ production. PHA and ionomycin (but not IL2) show significant synergy with PMA as a stimulus to LGL. In contrast, CD3+ T cells require two stimuli for high levels of IFN-γ production and not only are phorbol myristyl acetate (PMA) plus ionomycin or PHA synergistic, but in addition, IL2 and PHA demonstrate some synergy. Furthermore, we have found by fractionation of peripheral blood lymphocytes that IL2 induced IFN-γ production is associated with the LGL population and not T cells. These results indicate that with certain stimuli LGL may be the predominant source of IFN-γ from peripheral blood lymphocytes. Molecular analysis of the T cell receptor genes has shown that functionally active human and rat LGL lack α, β, and γ chain rearrangement or mRNA expression. Therefore, unlike in cytotoxic T cells, the T cell receptor is not involved in the

recognition of target cells by LGL. In addition the methylation state of the T cell receptor β-chain gene (T-β) DNA of T cells, LGL, B cells and monocytes to determine if methylation can be correlated with the reported transcriptional activity of this gene and to determine if this methylation pattern can be used as a marker for different leukocyte populations. By using the restriction enzyme isoschizomers, (Msp I and Hpa II), we found that T-β of T cells were highly unmethylated, those of B cells and monocytes are highly methylated and the methylation state of T-β of LGL is intermediate between that of T cells and B cells or monocytes. These results indicate that the methylation pattern of T-β can be a marker for T cells and LGL. The difference in methylation pattern of T-β between LGL and T cells and other leukocytes suggested that human peripheral blood LGL are distinct cell subsets. Studies regarding the alteration of DNA methylation and its functional significance are being conducted.

III. Antibody-Dependent Cellular Cytotoxicity (ADCC) by LGL with Mouse Monoclonal Antibodies

Studies have been completed to examine the potential role of ADCC in antitumor responses. A series of murine monoclonal antibodies against NK resistant melanoma and colon carcinoma cells were used to identify effector cells in peripheral blood which were capable of mediating ADCC against NK-resistant tumor targets. These results demonstrated that in the human, cells previously designated K cells which have LGL morphology mediated this activity. Additional studies revealed that the IgG-3 subclass of immunoglobulin functioned most efficiently in ADCC, and that the ADCC effector cell was regulated in vitro by IFN and IL2 in a manner similar to that previously described for augmentation of NK activity. Preclinical studies, in collaboration with Dr. J. Pearson, Experimental Therapeutics Section, are presently underway.

IV. Cytotoxicity by Cultured Cells

An in vivo xenograft model for human ovarian carcinoma has been used to perform adoptive cell therapy with various human effector cell populations. Human lymphoid and monocytoid effector cells were isolated and activated in vitro with rIL2 or IFN-γ. A significant extension of survival time in this ovarian carcinoma model could be achieved when IL2-activated LGL were transferred in animals bearing tumors. T cells activated with IL2 also induced a significant prolongation of survival of animals bearing the ovarian carcinoma. Monocytes with or without activation induced no significant prolongation of survival. Studies combining chemotherapy and monoclonal antibodies are presently underway.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09257-05 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Functional Activity of Large Granular Lymphocytes in Rats

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

PI:	C. W. Reynolds	Head, Cellular and Molecular Immunology Section	LEI, NCI
Others:	H. Fukui	Guest Researcher	LEI, NCI
	C. Cavalier	Bio. Lab. Technician	LEI, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Experimental Immunology

SECTION

Cellular and Molecular Immunology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The present series of studies have examined the functional activity of rat and human large granular lymphocytes (LGL), the population of cells known to mediate natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC). The adoptive transfer of LGL into rats with depressed NK/ADCC activity was shown to restore antitumor activity, as well as inhibit the development of syngeneic bone marrow stem cells. These results provide the first direct evidence for an important role for LGL in antitumor responses and in the control of bone marrow growth and differentiation. These results also suggest that the adoptive transfer of highly enriched LGL populations should be further considered as one potential immunotherapeutic regimen in cancer patients. In addition, protocols involving the depletion of host NK activity in bone marrow transplantation recipients should be further considered. In other experiments we have shown a number of differences in the organ, age and strain distribution between the NK and ADCC effector cell (killer cell) populations. Studies with the BRM, OK432, have shown this agent to augment NK activity and increase survival of tumor-bearing rats.

PROJECT DESCRIPTION

PERSONNEL

Craig W. Reynolds	Head	CMIS LEI NCI
Hiroyasu Fukui	Guest Researcher	CMIS LEI NCI
Corinne Cavalier	Bio. Lab. Technician	CMIS LEI NCI

OBJECTIVES

The objectives of this project are: (1) to directly investigate the role of large granular lymphocytes (LGL) in the in vivo resistance to tumor growth, metastasis and syngeneic bone marrow transplantation, (2) to compare the natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC) of LGL and 3) to study the effect(s) of biological response modifiers (BRMs) on NK activity and tumor immunity.

MAJOR FINDINGS

I. The Role of LGL in In Vivo Antitumor and Bone Marrow Transplantation Immunity

Previous studies had shown that rats treated with anti-asialoGM1 (asGM1) antibody showed a parallel decrease in NK activity, frequency of LGL in the spleen and peripheral blood, and clearance of tumor cells from the lungs; indicating that the antiserum-induced depression of NK activity was due to an elimination of most effector cells.

To further determine the possible role of LGL in tumor cell rejection in vivo, we studied LGL involvement in the inhibition of tumor cell metastases in the lungs. Animals treated with anti-asGM1 were found to have a greater than 10-fold increase in the number of lung metastases which developed following the i.v. injection of syngeneic MADB106 mammary tumor cells. Furthermore, the adoptive transfer of 3-10 million highly enriched LGL into NK-depressed animals 2 hours before tumor challenge partially or fully restored the ability of rats to inhibit the development of pulmonary metastases. These results are the first unequivocal evidence that LGL, which are highly enriched in NK activity, play an important in vivo role in the rapid elimination of circulating tumor cells with subsequent inhibition of metastasis.

Similar adoptive transfer studies in anti-asGM1 treated rats have also shown a significant role for NK cells in the inhibition of syngeneic bone marrow stem cell growth and differentiation. Anti-asGM1 treatment of lethally irradiated recipients increased the number of colony forming units in the spleen (CFU-S) following syngeneic bone marrow transfer. The adoptive transfer of LGL but not T cells into these irradiated/anti-asGM1 treated recipients significantly reduced the number of CFU-S seen. In addition, in vitro cytotoxicity assays using highly purified LGL and bone marrow stem cells showed significant lysis of the bone marrow cells. These results further emphasize the important in vivo role of NK cells in the control of syngeneic hematopoietic stem cell growth and differentiation.

III. Comparison of Rat NK and ADCC Activity

In the present studies, we have compared the distribution of NK and ADCC activities in rats with regard to: a) organ, b) strain, c) age, and d) Percoll density gradient fractionation. Appreciable NK and ADCC activities were observed in peripheral blood lymphocytes (PBL), splenic lymphocytes (SPL) and peritoneal exudate cells (PEC) but not in cell preparations from the lymph node, bone marrow and thymus. However, the ADCC reactivity in the PBL from F344, athymic nude, nude/+, Lewis, PVG/RTLRL and PVG/OLA rats, was consistently 3-10 fold higher than NK activity, while the PEC from the same animals showed appreciable NK activity but little ADCC. On a per cell basis, however, there was about the same NK and ADCC activity from the spleens of these rats. In contrast to the results in the above strains, the ADCC activity in WF/N rats was significantly lower in the PBL and SPL as well as PEC. Variations of NK and ADCC activity with age were also assessed in both a high ADCC (F344) strain and low ADCC (WF/N) strain of rats. In PBL or SPL from F344 rats, the level of ADCC but not NK activity increased with age. On the contrary, in WF/N rats the level of both NK and ADCC activities remained stable up to 35 weeks of age. To further investigate the mechanism for reduced ADCC activity in WF/N rats and to compare the cell populations which exhibit NK and ADCC activities, PBL and SPL from various strains of rats were fractionated by discontinuous Percoll density gradients. In all strains tested, the NK and ADCC activities were associated mainly with cells in the low-density fractions. These fractions also contained a high frequency of LGL and cells with receptors for the Fc portion of IgG (FcγR+ cell). In contrast, the low density PBL fraction from WF/N rats contained 50.8% LGL/<10% FcγR+ cells. These results suggest that both FcγR+-LGL and FcγR--LGL exist, and that the reduced ADCC activity in WF/N rats is due to a low frequency of FcγR+-LGL.

III. BRM Treatment and Effect on Tumor Growth

The BRM, OK432, was used to assess the effect of BRM treatment on the development of the host's antitumor immune response. F344 rats challenged i.p. with a lethal dose of the syngeneic MADB106 mammary carcinoma could survive more than 100 days when given a single dose of 5 mg/kg of OK-432 i.p. 1 day after tumor challenge. When examining the responsible effector mechanisms in this therapeutic model, two distinct effector phases distinguished by the number of tumor cells were evident. Phase I, 1-6 days following OK-432, resulted in a sharp decrease in tumor cell numbers and was related to the direct anti-tumor cytotoxicity of OK-432 and was coincident with an increase in the number of polymorphonuclear neutrophils (PMN). However, by day 6 a sharp increase in tumor cell numbers was again observed. Subsequently, a second phase of tumor cell destruction was observed 7-20 days following OK-432 and was reflected in a strong lymphocyte-mediated cytotoxicity response as well as the production of complement-dependent cytotoxic antibody against the MADB106 tumor cells. Using an antibody (R1-3B3) and complement depletion of cytotoxic PEC, the MADB106 killer cells appeared to consist of both R1-3B3- (non-T) and R1-3B3+ (T) cells, with most of the anti-MADB106 killing residing in the R1-3B3- cell population. The R1-3B3- killer cells were further defined as: 1) phenotypically asialo GM1+, 2) present in athymic nude rats, and 3) accompanied by some augmentation of YAC-1 killing (the prototype rat NK target), suggesting that some of these

R1-3B3- killer cells were typical NK cells. However, it was also observed that most of the R1-3B3- cells which killed MADB106 tumor cells were: 1) phenotypically or functionally different from either cytotoxic T cells or typical NK cells, 2) observed only in MADB106 tumor-bearing rats challenged with OK-432, 3) not present on day 1-2 following OK-432 injection, the time when YAC-1 killing was maximally augmented, and 4) present in high numbers in a secondary response following reinoculation of the MADB106 tumor cells into cured rats. The in vivo relevance and possible derivation of these various cytotoxic lymphocyte populations in the syngeneic tumor-bearing hosts is discussed. In conclusion, this study has demonstrated that the antitumor effects seen with OK-432 are due to a combination of sequential effector mechanisms leading to the eventual rejection of established tumor.

In collaboration with Dr. Robert Wiltrout (Z01 CM 09262-05 LEI) we also studied the augmentation of organ-associated NK activity by a variety of BRMs, including OK-432. The results from these studies demonstrate that BRMs induce a mononuclear cell infiltrate in many lymphoid and nonlymphoid organs. This infiltrate appears to correlate with the rapid appearance of organ-associated NK cells following BRM injection. These results suggest that the rapid infiltration of LGL into sites of inflammation may be an important factor in the subsequent development of mononuclear cell infiltration.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Drug Resistance in Cancer Cells

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

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Others:	W. E. Bere	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*

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☐ (a2) Interviews

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

The present studies examine the mechanisms by which cancer cells become resistant to cancer chemotherapeutic drugs. The role of glutathione S transferase (GST) in drug resistance has been investigated by the production of monoclonal antibodies (MoAb) to a novel GST isolated from adriamycin-resistant human breast carcinoma cells. These MoAbs demonstrate that this GST is immunologically related to placental glutathione transferase but not a GST isozyme isolated from liver. These MoAbs have also been useful in examining the distribution of GST in human tumor cells by flow cytometric analysis of lysolecithin-permeabilized cells and by immunofluorescence. Novel mechanisms of drug resistance have also been examined in human colon cancer cells by the use of DNA-mediated gene transfer to isolate genes that are able to confer a drug-resistant phenotype upon drug-sensitive cells. It is anticipated that these findings will have an impact on studies of the mechanisms of resistance of tumor cells to cytotoxic immunological mediators.

PROJECT DESCRIPTION

PERSONNEL

Craig Reynolds	Head	CMIS LEI NCI
William Bere	Biologist	CMIS LEI NCI

OBJECTIVES

The objectives of this project are: 1) to study the role of glutathione transferase (GST) in drug resistance and malignancy and 2) to search for novel mechanisms of drug resistance in tumor cells.

MAJOR FINDINGS

I. Monoclonal Antibodies to Glutathione Transferase

Monoclonal antibodies (MoAbs) have been produced to a novel GST isolated from an adriamycin-resistant human breast carcinoma cell line, MCF-7 ADr. Three mouse IgM MoAbs were isolated that reacted with GST isolated from the MCF-7 ADr cells and human placenta, but not GST from human liver. Thus, these MoAbs recognize an isozyme-specific epitope on the GST molecule. These MoAbs have been useful in examining the GST content of lysolecithin-permeabilized tumor cells including colon and breast carcinomas by flow cytometry. These MoAb did not: 1) immunoprecipitate GST, 2) inhibit GST enzymatic activity, or 3) react with GST in Western blot analysis. To evaluate the usefulness of GST as a tumor marker, these MoAbs are presently being used for the immunohistological analysis of human tumors in collaboration with Dr. P.G. Natali (Regina Cancer Institute, Rome, Italy).

II. Novel Mechanisms of Drug Resistance

Human colon carcinomas display an inherent resistance to most cancer chemotherapies. The basis for this resistance has not been delineated. In the present studies we have utilized DNA-mediated gene transfer to identify genes involved in the drug-resistant phenotype. The human colon carcinoma line, HT-29, was chosen for these studies as it displays a high in vitro resistance to most chemotherapeutic drugs and is used at FCRF in a human xenograft nude mouse model system to evaluate new chemotherapeutic regimens. High molecular weight DNA isolated from HT-29 was co-transfected with a selectable marker into NIH3T3 cells. Transfectants were then selected for growth in the presence of either single drugs or drug combinations. Presently, multiple rounds of transfection are being used to isolate individual human genes which will be isolated from gene libraries constructed from the transfectants by screening with probes to repetitive human DNA sequences.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09262-05 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Antitumor Effects of rIL2-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, Experimental Therapeutics Section	LEI, NCI
Others:	R. L. Hornung	Biotechnology Fellow	LEI, NCI
	E. Ayroldi	Visiting Fellow	LEI, NCI
	P. L. Urias	Chemist	LEI, NCI
	M. E. Gruys	Biologist	LEI, NCI

COOPERATING UNITS *(if any)*

Preclinical Screening Laboratory, Program Resources, Inc., NCI-FCRF, Frederick, Maryland 21701

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRF, 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.)*

Simultaneous intravenous and intraperitoneal administration of adoptive chemoimmunotherapy (ACIT) to mice bearing advanced (Stages II or III) murine renal cancer (Renca) was able to render 80% of these mice disease free, while administration of ACIT by either route alone was unsuccessful. These observations demonstrate that the antitumor effects of AIT are increased when used in conjunction with chemotherapeutic drugs and emphasize the need to efficiently deliver the ACIT to all sites of tumor growth. More recent studies have demonstrated that the investigational drug Flavone-8-acetic acid (FAA) potently augments natural killer (NK) activity the spleen, liver, lungs and peritoneum. Since NK cells can serve as precursors for lymphokine activated killer (LAK) cells, additional studies were performed to determine whether FAA and recombinant interleukin 2 (rIL2) could have additive or synergistic effects for treatment of Renca. A single dose of FAA (250 mg/kg) followed by four daily i.p. doses of rIL2 (10,000 U to 100,000 U/day) cured 50-80% of mice bearing advanced Renca, while FAA or rIL2 alone were unable to render any mice disease free. Studies are in progress to determine the mechanism(s) by which FAA augments NK activity and cooperates with rIL2 for treatment of Renca. Additional studies are designed to determine the mechanism(s) by which biological response modifiers (BRMs) induce augmented NK activity and an accumulation of NK-active large granular lymphocytes (LGL) in the liver. These experiments have utilized the bone-seeking isotope strontium-89 (89-Sr) to preferentially eliminate bone marrow cellularity prior to the administration of BRM. By this regimen we have determined that 89-Sr pretreatment inhibits the BRM-induced augmentation of liver-associated leukocytes by 40-60%. These results suggest that much of the increase in liver-associated NK activity induced by BRM results through an accumulation of NK cells recently derived from bone marrow progenitors. These observations are currently being used to determine the contribution of NK cells to BRM-induced antimetastatic effects in nonlymphoid organs.

PROJECT DESCRIPTION

PERSONNEL

Robert H. Wiltrout	Head	ETS	LEI	NCI
Ronald L. Hornung	Biotechnology Fellow	ETS	LEI	NCI
Emira Ayroldi	Visiting Fellow	ETS	LEI	NCI
Patricia L. Urias	Chemist	ETS	LEI	NCI
Eilene Gruys	Biologist	ETS	LEI	NCI

OBJECTIVES

To study the ability of chemotherapeutic drugs to complement the antitumor effects mediated by adoptively transferred rIL2-stimulated cytotoxic lymphocytes and/or recombinant interleukin 2 (rIL2) in the treatment of established murine renal cancer, and to determine the mechanism by which biological response modifiers (BRM) modulate natural immunity in nonlymphoid organs.

1. To determine the extent to which chemotherapy and adoptive immunotherapy (AIT) complement each other for the treatment of murine renal cancer.

2. To determine whether the novel investigational agent Flavone-8-acetic acid (FAA) functions as a BRM.

3. To determine whether FAA and rIL2 can exert additive or synergistic antitumor effects against established murine renal cancer.

4. To isolate, and characterize the precursor cells which give rise to lymphokine-activated killer (LAK) cells in the mouse.

5. To study the mechanism(s) by which BRMs induce an increase in NK activity and total number of large granular lymphocytes (LGL) in the liver.

There are two major areas of study being developed. First, a major focus of the research is on the development and utilization of experimental models of adoptive chemoimmunotherapy (ACIT) and chemoimmunotherapy (CIT) for the treatment of both primary tumors and their metastases. These studies are performed using the murine renal carcinoma designated Renca. The studies are also designed to investigate the potential of different chemotherapeutic drugs for use in ACIT and CIT and the mechanism(s) by which ACIT and CIT are effective. Special emphasis is placed on the nature of the responder and effector cells modulated by rIL2, as well as the contribution of the recipient's immune system to observed therapeutic effects. The second major area of research focus is the mechanism(s) by which BRMs augment natural immunity in vivo. Particular areas of study include the identification of cell types modulated, the degree of anatomical compartmentalization associated with those effects, the in vivo dynamics of LGL localization, and factors which regulate these changes. The focus of these studies during the past year has been to determine the mechanism by which BRM-induce augmented natural killer (NK) activity and LGL number in the liver. Additional studies have been performed to determine the contribution of chemotactic responses in the regulation of BRM-induced localization of NK cells.

Extensive studies have been performed to determine the therapeutic efficacy of utilizing adoptive immunotherapy (AIT) by rIL2-stimulated lymphocytes and exogenous rIL2 in combination with chemotherapeutic drugs for the treatment of murine renal cancer. These studies have shown that AIT or chemotherapy by doxorubicin hydrochloride (DOX) was unable to cure mice bearing Stage II and III renal cancer, while bicompartmental (i.v. + i.p.) adoptive chemoimmunotherapy cured approximately 70% of these mice. We have also studied the nature of the precursor cells which give rise to both activated NK cells and LAK cells. The results show that both activated NK cells and LAK cells arise from an asialo GM1+, LYt2-, L3T4- precursor, a phenotype consistent with the NK cell. Because of the relationship of NK cells to LAK activity and therefore perhaps to the LAK-associated antitumor effects related to rIL2 treatment, we have investigated the ability of the investigational agent FAA to modulate NK activity as well as its ability to mediate antitumor effects when used in concert with rIL2. These studies demonstrated FAA to be a potent augmenter of systemic NK activity at doses of 250 mg/kg and 125 mg/kg. These doses have been previously shown to induce antitumor effects against a variety of murine solid tumors which have proven refractory to most drugs. Further studies demonstrated that FAA and rIL2 function synergistically in the treatment of murine renal cancer such that 50-80% of Renca-bearing mice could be rendered disease free by this regimen while no mice were cured by FAA or rIL2 alone. Most interestingly this therapeutic activity was achieved without the requirement for adoptively transferred LAK cells and could be performed using doses of rIL2 which were nontoxic (1000 U to 30,000 U/mouse/day). Further studies are in progress to determine the mechanism(s) for these effects.

Studies are underway to determine the ability of BRMs to augment NK and macrophage-mediated cytotoxicity and induce antimetastatic effects in nonlymphoid organs, which are often sites for development of metastases during the progression of human cancer. The liver is being used as a model organ. Current studies are focused on determining the mechanism by which BRMs induce an accumulation of NK-active LGL in the liver. Various types of BRM can augment NK activity in lymphoid and non-lymphoid organs. This augmentation of NK activity is often accompanied by an accumulation of blood-borne leukocytes, including NK-active LGL. Pretreatment of mice with 89-Sr which eliminates bone-marrow derived NK progenitors, also inhibits the augmentation of liver-associated NK activity by the pyran copolymer, maleic anhydride divinyl ether (MVE-2). The augmentation of liver-associated NK activity by single or multiple doses of MVE-2 is inhibited by 82% or 85%, respectively, following a single i.p. pretreatment of 125 uCi 89-Sr/mouse. Similarly, the accumulation of leukocytes following MVE-2 pretreatment is inhibited by 40-60% by this regimen of 89-Sr administration. In contrast, bone marrow cellularity is depleted by >95% at 3-6 days, while spleen cellularity and baseline (no BRM injection) splenic and hepatic NK activity are not significantly affected by 89-Sr pretreatment. Further studies have also demonstrated that splenectomy prior to the administration of MVE-2 does not significantly inhibit the infiltration of leukocytes or augmentation of liver-associated NK activity. The present results demonstrate that the augmentation of liver-associated NK activity by MVE-2 occurs primarily through an accumulation of NK cells recently derived from bone marrow progenitors. Overall, these studies should provide insight into how BRM redistribution of leukocytes is regulated and contribute to our understanding of the mechanisms by which BRMs induce antimetastatic effects in nonlymphoid organs.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09288-02 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Chemoimmunotherapeutic Modalities Against Established Tumors and Metastases

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, Exp. Therap. Section	LEI, NCI
	D. L. Longo	Associate Director	BRMP, NCI
	J. R. Ortaldo	Chief,	LEI, NCI
	H. Hirte	Guest Researcher	LEI, NCI
	H. Shinomiya	Guest Researcher	CRB, NCI
	T. E. Hamilton	Senior Investigator	MB, NCI
	D. Fitzgerald	Senior Investigator	LMB, NCI

COOPERATING UNITS *(if any)*

LAK Section, Program Resources Inc. (S. Beckner)

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	2.25	2.00

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 major focus of this project has been to develop chemoimmunotherapeutic approaches to the treatment of a human ovarian (OVCAR) and colon (Ht-29) tumors in nude mice. The intraperitoneal (i.p.) administration of optimal doses of cyclophosphamide (CY) (250 mg/kg) and cis-platinum (5 mg/kg) 21 days after the injection of 5 million OVCAR cells resulted in a 4 week remission period, with no demonstrable ascites, prior to relapse and death due to localized peritoneal disease (80 to 110 days). Untreated mice routinely expire within 55 to 65 days. Treatment of mice with optimal doses of CY (200 mg/kg), Flavone-8-acetic acid (FAA) (200 mg/kg), melphalan (.25 mg) and doxorubicin hydrochloride (DOX) (.2 mg) 7 days after injection of HT-29 ascites resulted in MSTs of 46.0, 50.0, 52.0 and 64.0 days, respectively as compared to a MST of 34.0 days exhibited by the untreated group. The concomitant administration of WR-2721, a chemoprotective agent, allowed the safe delivery of higher doses of CY (500 mg/kg), FAA (300 mg/kg) and melphalan (.5 mg). This ability to administer higher amounts of chemotherapeutic drugs translated into enhanced antitumor efficacy as determined by longer remission periods and increased MSTs of mice bearing Ht-29. The administration of multiple i.p. treatments of human LAK cells 30 million and rIL2 (20,000 U) beginning 3 weeks into the remission period induced by cytoreductive therapy against OVCAR resulted in a MST of 121.0 days before eventual relapse and death of all mice. In contrast, animals treated with LAK cells or rIL2 alone exhibited MSTs of 105.0 and 97.0 days, respectively. A similar therapeutic approach is underway where drug treated mice have received multiple i.p. injections (1 μg) of monoclonal antibody (MoAb) (OVB-3) specific for ovarian cancer. Experiments have also been performed using OVB-3 conjugated to Pseudomonas exotoxin (P.E.). All mice treated with OVB-3 are presently free of disease (70 days). Finally, OVB-3 (P.E.), activated human monocytes and specific lymphokines are being tested against Ht-29 ascites tumor.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

John W. Pearson	Microbiologist	ETS	LEI	NCI
Robert H. Wiltrout	Head	ETS	LEI	NCI
Dan L. Longo	Associate Director		BRMP	NCI
John R. Ortaldo	Chief		LEI	NCI
Holger Hirte	Guest Researcher	LDS	LEI	NCI
Hiroto Shinomiya	Guest Researcher	CRB	BRB	NCI
Thomas E. Hamilton	Senior Investigator		MB	NCI
David Fitzgerald	Senior Investigator		LMB	NCI

OBJECTIVES

The aim of this project is to test the effectiveness of various immunotherapeutic modalities for the treatment of established primary and experimentally induced metastatic tumors during remission periods induced by cytoreductive therapy. The models used include human ovarian (OVCAR) and colon (Ht-29), carcinomas xenografted into nude mice. The specific objectives of this project are as follows: (1) to identify clinically active drugs, as well as optimal doses, that will induce a state of remission in animals having established ovarian or colon disease, (2) to ascertain the therapeutic effectiveness of adoptive immunotherapy of human tumor xenografts by adoptively transferred human cytotoxic lymphocytes following cytoreductive therapy and (3) to determine the therapeutic efficacy alone, or in conjunction with drugs, of cytokines or immunotoxins coupled to specific monoclonal antibodies (MoAb) against local tumor growth (s.c. or i.p.) or against experimentally induced pulmonary or liver foci.

MAJOR FINDINGS

I. Chemotherapeutic Treatment of Human Tumor Xenografts in Nude Mice

Since the major objective of this project is to investigate the possible therapeutic benefits of combined chemoimmunotherapy, a major effort was to define optimal regimens of chemotherapy which could induce remissions in the growth of the OVCAR and Ht-29 tumors xenografted into nude mice. The drugs of choice utilized against the OVCAR were cyclophosphamide (CY) and cis-platinum. Inoculation of 5 million tumor cells, i.p., into mice results in localized peritoneal ascites and carcinomatosis. Animals die between 55 and 65 days. The i.p. administration of maximal doses of cytoxan (250 mg/kg) and cis-platinum (5 mg/kg) 21 days post tumor cell inoculation, a time when physical signs of ascites are grossly apparent, resulted in a remission period characterized by a lack of visible ascites. The duration of these remissions was routinely about 4 weeks with subsequent relapse and death of all treated mice in 80-110 days. The drugs of choice to screen for a therapeutic effect against the Ht-29 were CY, Flavone-8-acetic acid (FAA), melphalan and doxorubicin hydrochloride (DOX). Administration of 3 million Ht-29 cells, i.p., to nude mice results in a localized peritoneal ascites with all animals dying from the disease 30 to 38 days post tumor cell inoculation. Recent studies have revealed that the i.p. administration of optimal doses of CY (250 mg/kg), FAA (200 mg/kg), melphalan

(.25 mg) and DOX (.2 mg) to mice injected 7 days earlier with Ht-29 resulted in median survival times (MST) of 46.0, 50.0, 57.0 and 64.0 days, respectively. Untreated mice exhibited a MST of 34.0 days. With all drugs, there was a brief remission period (7-10 days) characterized by the lack of grossly visible ascites. Therefore studies were undertaken to attempt to increase the amount of drug that could be tolerated for all of the above mentioned agents. WR-2721, a chemoprotective agent, was used in conjunction with the drugs in hopes of increasing the amount of drug tolerated by the mice and thereby to also increase the therapeutic effects against the Ht-29 colon tumor. Toxicology investigations revealed that the i.p. administration of 400 mg/kg of WR-2721 one half hour before drug delivery protected normal mice from otherwise lethal doses of CY (500 mg/kg), FAA (300 mg/kg), and melphalan (.5 mg). WR-2721 had no protective effect against a lethal dose of adriamycin (.5 mg). Subsequent studies demonstrated that indeed WR-2721 when administered in conjunction with an otherwise lethal dose of CY or melphalan to mice 7 days after Ht-29 inoculation resulted in increases of MSTs to 69.0 and 62.0 days, respectively. This translates into a 50% increase in MST for these drugs. In addition, a period of remission of approximately 3 weeks was obtained before eventual relapse and death of all treated mice. WR-2721 was found to have no additive effect when used in combination with a lethal dose of FAA against the colon tumor (MST=53.0). Untreated mice died within a period of 34 to 38 days. Various forms of immunotherapy will now be utilized following "debulking" therapy against the ovarian and colon tumors xenografted in nude mice.

II. Chemoimmunotherapy of OVCAR in Nude Mice

Studies were designed to explore the therapeutic effect of human lymphokine activated killer (LAK) cells against OVCAR following a CY or cis-platinum-induced remission in athymic mice. Three weeks following "debulking" therapy, animals were inoculated i.p. with LAK cells 30 million and/or with rIL2 (20,000 U) daily for 3 days. Mice that received CY or cis-platinum alone exhibited an MST of 87.0 days as compared to a MST of 60.0 days for the untreated control group. Groups of animals treated with LAK or rIL2 alone demonstrated increases of MSTs of 105 and 97 days, respectively with no long-term survivors. The combination of LAK cells and rIL2 resulted in a further increase in MST of 121.0 days before relapse and eventual death of all mice. Similar studies are underway where mice that received CY and cis-platinum were treated with a specific MoAb (OVB-3) reactive with antigens or the surface of OVCAR. Studies are also being performed with OVB-3 which has been conjugated to an immunotoxin made from Pseudomonas exotoxin (P.E.). Three weeks following drug therapy, mice received i.p. 1 ug of OVB-3 or OVB-3 (P.E.) daily for a total of 6 treatments. This study suggests that mice treated with OVB-3 and OVB-3 P.E. will survive longer than the controls. More extended observations will be required to determine whether the MoAb can enhance the effects of chemotherapy.

III. Adoptive Immunotherapy of Human Tumor Xenografts in Nude Mice

It was reported that human recombinant alpha interleukin demonstrated therapeutic activity in patients with ovarian cancer. Studies were designed whereby alpha interferon was administered i.p. against an early OVCAR tumor. Animals inoculated i.p. with 5 million ascites cells were treated with alpha interferon 100,000 U for 5 days starting 3 days post tumor cell inoculation.

While the untreated control group exhibited a MST of 55.0 days all treated mice are surviving 80 days post tumor cell inoculation showing no physical signs of disease. Plans are underway to incorporate alpha interferon following "debulking" therapy in the OVCAR model. A series of experiments are currently underway exploring the therapeutic effect of various biologicals against the Ht-29 colon tumor in nude mice. Animals inoculated i.p. with 3 million ascites cells followed by the administration of alpha interferon 100,000 U alone/or with rIL1 (250 ng) daily for 5 days beginning 3 days post tumor cell injection are currently under observation. Currently, 70 days after tumor injection, 70% of the mice treated with alpha interferon alone or in combination with rIL1 are surviving. In contrast, rIL1 alone was ineffective against the colon tumor as all animals expired in a range of 26 to 39 days, which is similar to that observed in the untreated control group.

A similar approach is underway utilizing OVB-3 (P.E.) immunotoxin alone and/or in combination with alpha interferon. Likewise, activated human monocytes are currently being administered to mice bearing an early ascites tumor. Eventually, studies will be undertaken to combine chemotherapy with biologics that have shown a therapeutic effect against the colon tumors xenografted in nude mice.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CM 09259-05 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Characterization and Differentiation of NK Cells and Lymphocyte Subsets

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

PI:	B. J. Mathieson	Head, Leukocyte Differentiation Section	LEI,	NCI
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	R. H. Wiltrout	Head, Experimental Therapeutics Sect.	LEI,	NCI
	J. Ortaldo	Chief	LEI,	NCI
	H. Hirte	Guest Researcher	LEI,	NCI
	A. Wilt	Bio. Lab. Tech.	LEI,	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc. (S. Giardina, M. Beckwith, C. Moratz, W. Bohn, R. Overton); Memorial Sloan-Kettering Cancer Center, New York City (F.-W. Shen)

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Leukocyte Differentiation Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Monoclonal antibodies (MoAbs) have been generated or modified to study the origin, differentiation, and potential function of naturally-occurring murine cytotoxic leukocytes. A rat MoAb (4D11) has been generated against a novel cell surface antigen on mouse large granular lymphocytes (LGL) with natural killer (NK) activity. This antigen, LGL-1, can be detected on splenic or liver-derived LGLs from most strains of mice but not on other types of lymphocytes or hematopoietic populations. Complement-mediated lysis with 4D11 depletes >50% of the NK activity in NK-enriched cells. In addition, cells electronically sorted for LGL-1 expression are enriched for NK activity. LGL-1 can be detected on populations with lymphokine-activated killer (LAK) activity, but it appears that LGL-1 is not expressed on all precursors or effectors of LAK activity. IgM isotype MoAbs are normally generated in Epstein-Barr virus-infected human B cell cultures, thus we examined techniques to generate and isolate more biologically useful IgG isotype switch variants of MoAbs to cell surface antigens. IgG variants of IgM anti-Qa-5, a murine MoAb recognizing NK cells in some mice, were selected to determine whether IgG MoAbs might be more useful than the parental IgM. However, the IgG variant MoAbs failed to bind Qa-5 antigen with sufficient affinity to pursue other studies. A clonal variant producing both IgM and IgG1 was also isolated, which suggested that the sequence for isotype switching may not be parallel to the genomic configuration for the IgH genes. Chemical modification of MoAbs to produce heterobifunctional molecules has been undertaken. Two 2-iminothiolane derivatives of a melanoma-specific MoAb have been produced and compared with SPDP linkage of biological response modifiers. Finally, MoAbs to fos and raf oncogene proteins have been generated and characterized. Studies on the interaction of oncogene products in normal activation and differentiation of various leukocyte subsets and the role of different oncogene products in malignant transformation are now being done.

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

PROJECT DESCRIPTION

PERSONNEL

Bonnie J. Mathieson	Head	LDS	LEI	NCI
Llewellyn Mason	Microbiologist	LDS	LEI	NCI
Robert Wiltrout	Head	ETS	LEI	NCI
John Ortaldo	Chief		LEI	NCI
Alan Wilt	Bio. Lab. Tech.	LDS	LEI	NCI
Holger Hirte	Guest Researcher	LDS	LEI	NCI

OBJECTIVES

The major objectives of this project are: 1) to identify and characterize the cellular phenotype of leukocytes that have natural killer (NK), or lymphokine-activated killer (LAK) activity, 2) to study the origin, differentiation, development and activation of these cells in vivo and in vitro, 3) to devise new reagents that can potentially target the effects of activated leukocytes at the site of tumors in vivo, and 4) to develop reagents that detect oncogene proteins that might be involved in activation or functional differentiation of subsets of leukocytes.

Our specific aims are to: (a) acquire and develop a panel of MoAbs and other reagents with selective or differential reactivity against mouse NK cells, (b) develop enrichment and selection techniques to isolate subsets of precursors and differentiated cells from various tissues, (c) characterize the isolated precursors of augmented NK and LAK cells from spleen, liver, or bone marrow by phenotype, growth requirements in vitro and pattern of cytotoxicity on different tumor cell lines and normal lymphoid targets, (d) compare such precursors with early thymocytes and myelomonocytic cells in vitro and in cell transfer studies with congenic mice, and (e) determine whether immature thymic cells can be shifted from conventional T cell differentiation toward differentiation into LGL with cytotoxic activity.

METHODS EMPLOYED

Animals were obtained from the Animal Production Area of the FCRF. C57BL/6 (B6), B6-Ly-5.2 congenic mice and BALB/cAnN animals were used routinely for animal studies. Cell lines were maintained for antibody production or cytotoxic cell targets by sterile culture techniques. When necessary, samples of human blood or bone marrow cells were obtained through the Clinical Research Branch.

To characterize NK cells and LAK cells in the mouse, we used MoAbs for the mouse that detect: 1) Ly-5, which is expressed on all leukocytes including NK cells; 2) The T cell specific antigens Ly-2 (CD8) and L3T4 (CD4) as well as Ly-1 and Thy-1 which are expressed on some non-T cells; 3) Qa-5, an MHC-associated, class I antigen expressed preferentially on NK and T cells; 4) asialo GM1 (asGM1) and Fc receptor, markers that have been associated with NK cells in mice and other species. MoAbs and other serological reagents for detection of surface antigens obtained from commercial sources or through collaborative agreements, titered and tested for appropriate reactivity.

I. Subset Enrichment Methods

Nylon wool nonadherent (NW-NA) cells from spleen or liver were subjected to Percoll density gradient fractionation to enrich LGL in the low density region of the gradient. The enriched fraction from spleen contains about 10-30% LGL in mice in contrast to the high level of purification seen for PB cells from humans or rats. Anti-Ig plate adherence was used to further remove contaminating B cells that interfered with the immunofluorescence analyses. Anti-Ly-2 and anti-L3T4 MoAb were used to further select and/or deplete T cells by indirect anti-Ig coated plate separations of mouse cells and facilitated complement (C)-dependent lysis was used where necessary.

Mouse liver interstitial cells that contain a high proportion of LGL (See Robert Wiltrout, project # Z01 CM 09262-05 LEI) were obtained by collagenase digestion of minced perfused livers from MVE-2, Poly IC:LC, or C. parvum-treated animals. These cells were further purified by removal of NW adherent cells and Percoll density enrichment. Immature thymocyte precursors (dLyl cells) were prepared as described in the accompanying project (Z01 CM 09282-03 LEI) by sequential MoAb + C lysis and removal of dead cells.

T lymphocyte subsets and NK cells were purified prior to culture in IL2 for identification and characterization of LAK cells. Purification methods rely on anti-Ig plate adherence of selected specific T subsets treated with MoAb to T cell markers and a final step using selective C-dependent elimination.

II. Cell Surface Phenotype

Phenotype of the isolated cell populations was determined by one of three methods: a) Flow cytometry analyses (FCA) to quantify cell surface antigen expression detected by immunofluorescence (IF) were performed on an Ortho Cytofluorograph. b) Cytotoxic elimination experiments with MoAb + C served two purposes: 1) To directly assess the phenotype of the eliminated cells relative to the level of NK or LAK function, 2) To eliminate unwanted or irrelevant cell subsets in enrichment methods for
spleen, blood and bone marrow before their use in IF or functional analyses. c) For visual assessment of antigen expression on morphologically identified cells, protein A dependent rosette methods were employed.

III. MoAb Production

Hybridomas producing Ig were obtained by fusion of immunized mouse or rat spleen cells with drug sensitive murine myeloma cells. After selection and expansion in vitro, ascites of hybridomas of interest were produced in nude mice or MoAbs were produced in vitro. Ig from ascites or culture supernatants was purified by sodium sulfate fractionation and column chromatography. Isotype switch variants were obtained by sequential subline selection from decreasing numbers of cells and cloned by limiting dilution.

MAJOR FINDINGS

I. Murine Precursors of NK Activity

Manipulation of lymphoid precursor populations from murine BM or other populations can be informative where relationships between both precursors and functional effectors can be assessed with genetic markers. Therefore, we have continued to develop methodology to identify, isolate, monitor, and assess cells with potential natural cytolytic capacity. We have also continued comparative phenotypic and functional analysis of NK cells, LAK cells, and T cells.

In other studies, we devised a stepwise selection procedure to isolate different murine lymphocyte subsets contained within the spleen. This procedure involves depletion of B cells and macrophages by nylon wool, followed by selective adherence of MoAb coated T cells to anti-Ig coated Petri dishes. Finally, the non-T, non-B cells are further depleted by MoAb + C lysis to enrich for LGLs and NK cells. The separated populations were then compared phenotypically and functionally (Wiltrout project #Z01 CM 09262-05 LEI) before and after culture in IL2 to determine what relationship exists between T cells, NK cells and LAK. These data indicate that the non-T cell population enriched for asGM1-bearing cells, contains virtually all of the NK activity before culture and is responsible for nearly all of the LAK activity after IL2 culture for 3-5 days. In contrast, Ly-2+ T cells which can proliferate in the IL2 are only marginally effective in producing LAK activity. Taken together, the murine and other human data strongly suggests a non-T origin of LAK cells.

Because of the high toxicity but extensive therapeutic potential of several immunomodulators known to activate LAK or NK cells and macrophages, a number of approaches have been used to link immunomodulators to anti-tumor MoAbs to allow targeting of their biological activities to tumors. Two novel 2-iminothiolane derivatives (2-pyridyl-disulfide-2-iminothiolane and 3-carboxy-2-nitrophenyl-disulfide-2-iminothiolane) were developed and should prove useful for linking immunoregulatory peptides and proteins to MoAbs because they preserve the charge at the site of chemical linkage. The heterobifunctional linker SPDP were successfully attached to both IL2 and IFN-γ and these preparations retain their biological activity after linker modification and after being linked to the melanoma-specific MoAb 9.2.27. SPDP was also linked to the peptides Tuftsin and FK-565 and these are also undergoing testing for activity after modification. Lastly, muramyldipeptide (MDP) has been linked directly to 9.2.27 by making an active ester derivative through its carboxyl group using dicyclohexyl-carbodiimide and N-hydroxy-succinimide. Up to 13 molecules of MDP per molecule 9.2.27 were attached with preservation of specific MoAb binding. The MDP conjugates will undergo testing for ability to target tumoricidal activity of macrophages.

II. Characterization of NK Cells Using Monoclonal Antibodies

No NK-specific antibodies have been available to study the lineage and differentiation of NK cells. Therefore several MoAbs were generated to study the origin differentiation, and potential function of naturally-occurring cytotoxic lymphocytes. By blocking the response to common lymphocyte antigens, rat MoAbs were raised to mouse LGL. In particular, A rat MoAb (4D11) was

generated that appears to recognize a non-allelic determinant on NK-active cell populations. The 4D11 antibody recognizes a novel cell surface antigen, and expression is correlated with the presence of LGLs in B6 mice and related congenics. Immunofluorescence studies indicate that this antigen is expressed on LGLs of most strains of mice but it is not detected on thymocytes, T or B lymphocytes or other hematopoietic populations. Antibody-dependent, complement-mediated lysis depletes 50 to 80% of the NK activity in leukocyte populations enriched for NK cells. Cells positively selected by electronic sorting of the 4D11+ leukocytes, contain 50 to 60% LGL and are enriched 25 to 60 fold in their lytic activity against YAC target cells. Preliminary studies indicate that this NK antigen is not expressed on all precursors and effectors of LAK or augmented NK activity, nor on CD3+ mouse lymphocytes from thymus or peripheral lymphoid tissues.

Qa-5 is an antigen detected on all NK cells, and defined by an IgM-secreting murine hybridoma. This MoAb completely abrogates NK activity from MoAb + C treated effector cells from normal or activated spleen and livers in vitro. We were interested in utilizing anti-Qa-5 in vivo as an alternative to heterologous, rabbit anti-asialo GM1 (asGM1) as a reagent for depleting resident NK cells from congenic mice which are to be reconstituted with donor BM cells. When this was attempted with the IgM anti-Qa-5 reagent in vivo, only a limited anti-NK effect was detected, possibly due to a rapid clearance of the IgM antibody from circulation. Furthermore, because human MoAbs of IgM isotype can be readily generated to various antigens in Epstein-Barr virus-infected cultures of human B cells, it was of interest to examine the potential usefulness of techniques to generate and isolate isotype variants to known cell surface antigens. To address this problem, we used the process of sequential sublining to select and clone anti-Qa-5 hybridoma cells which have spontaneously switched to production of IgG isotypes. We successfully isolated switch variants of the IgG2a, IgG2b, and IgG1 subclasses. In addition, a separate clone that secretes both IgG1 and IgM simultaneously was isolated. This has been confirmed by two-color immunofluorescence. This type of double producing variant suggests that the sequence of plasma cell differentiation for different isotype expression is not collinear with the Ig gene organization, and that the deletion of intervening IgH sequences may not be required for switching to occur.

We also determined that pretreatment of the parent IgM secreting clone with a murine erythroleukemia differentiating agent, hexamethylene-bis-acetamide (HMBA), enhances the number of detectable switch variants approximately 3 fold. When tested for anti-Qa-5 specificity by binding to C57BL/6 lymph node cells, we found that none of the switch variants maintained easily detectable levels of binding to Qa-5 + cells. We demonstrated that when purified by protein-A Sepharose, and used in 50X excess, the IgG2 isotype switch variant was able to competitively-inhibit binding of the parent IgM. In addition, anti-Qa-5 IgM lost its ability to bind when reduced to monomeric subunits. Therefore, we conclude that the loss of antigen-specific binding by the isotype switch variants is most likely due to a decrease in affinity when the IgM pentameric structure is lost.

III. Characterization of Oncogene MoAbs and Expression in Activated or Malignant Cells

To elucidate the role of oncogenes in cellular differentiation and malignant transformation, murine MoAb were generated to the fos and raf oncogene products. In collaboration with several investigators in the BRMP, anti-fos MoAbs were developed using synthetic peptides representing two distinct molecular domains. One MoAb, 2G9C3, against a twenty-four amino acid consensus region of both v-fos and c-fos detects three proteins with molecular weights of 55,000, 44,000 and 42,000 by immunoblotting. As expected, immunohisto-chemical staining of FBJ virus-infected cells revealed intense, nuclear staining. However no cell surface membrane expression of fos was detected. After membrane permeabilization by a brief exposure to lysolecithin, it was possible to specifically detect internal fos by immunofluorescence flow cytometry.

There is evidence which suggests that the ras oncogene located on the cytoplasmic face of the cell membrane may influence the expression of fos in the nucleus. In conjunction with Drs. A. Carbone and Luigi Varesio we have begun investigating the expression of the fos oncogene in fibroblast cell lines transformed by the ras oncogene. Our evidence suggests that while fos protein can be induced by PMA in normal as well as N-ras and K-ras transformed lines, it is constitutively expressed in H-ras transformed lines at a level which is not modifiable by PMA.

The anti-raf MoAb, MUR 1.4, was produced against a 30 K v-raf protein produced in E. coli. A 30 K protein as well as a series of breakdown products are detectable by immunoblot analysis. Preliminary evidence suggests that MUR 1.4 is capable of immunoprecipitating a 44 kD c-raf protein from cells transfected with multiple copies of the raf gene. Collaborative studies are in progress with Drs. Mark Smith and Hsiang-Fu Kung to determine the effect upon cell division and morphology following microinjection of MoAbs into adherent cell lines transformed with raf as well as other oncogenes. In this way it should be possible not only to investigate the potential involvement of raf in tumorigenesis but also to determine its location in the sequence of gene interactions leading to malignancy. Furthermore, application of the lysolecithin technique has enabled us to detect raf protein in a monocytic cell line transformed by a myc/raf viral construct. Thus, cellular expression levels in activated cells may be monitored at the protein level by this technique and we plan to use this approach to study oncogene levels in different subsets of leukocytes.

PUBLICATIONS

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Peppoloni, S., Mathieson, B.J., Herberman, R.B., Overton, R.W., and Gorelik, E.: In vivo resistance of secondary anti-tumor immune response to cyclophosphamide: Effects on T-cell subsets. Cancer Immunol. Immunotherap. 24: 49-57, 1987.

Salup. R.S., Mathieson, B.J., and Wiltrout, R.H.: Precursor phenotype of lymphokine-activated killer cells in the mouse. J. Immunol. 138: 3635-3639, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09282-03 LEI

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Potential Differentiation Capacity of Thymocytes

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

PI:	B. J. Mathieson	Head, Leukocyte Differentiation Section	LEI,	NCI
Other:	T. Gregorio	Bio. Lab. Tech.	LEI,	NCI
	L. Mason	Microbiologist	LEI,	NCI
	J. Wine	Bio. Lab. Tech.	LEI,	NCI
	K. Matsushima	Visiting Associate	LMI,	NCI
	F. Ruscetti	Head, Lymphokines Section	LMI,	NCI
	H. A. Young	Expert	LMI,	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc. (K. McConville-Komschlies, R. Overton, L. Finch); Arthritis and Rheumatism Branch, NIAMSD, (W. C. Gause); Memorial Sloan-Kettering Cancer Center (F.-W. Shen).

LAB/BRANCH

Laboratory of Experimental Immunology

SECTION

Leukocyte Differentiation Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.0	0.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The differentiation capacity of isolated subsets from normal mouse thymus has been examined in vivo and in vitro. Intravenous (i.v.) experimental cell transfers using congenic mice, have previously determined that an immature, intrathymic subset of adult mouse thymocytes are dull Lyl+ (dLyl) cells lacking both Ly-2 (CD8) and L3T4 (CD4) cell surface expression. These dLyl cells are contain committed thymocyte progenitors with limited capacity for regeneration. Thymocyte precursors have also been demonstrated in bone marrow by i.v.transfer, but this route restricts the "re-homing" of more mature cells back to the thymus. Several additional, more differentiated, subsets of thymic cells have now been transferred intrathymically (i.t.). These include the Ly-2+,L3T4+ blast population, and L3T4+ or Ly-2+ thymocytes. Yet, not all "immature" thymocytes are capable of transfer and further differentiation. Attempts to transfer early fetal thymocytes (day 13-15 of fetal gestation) have been relatively unsuccessful, whereas both Ly-2+,L3T4+ and dLyl cells, present at 16-day, yield donor-derived cells. Furthermore, the double positive blasts yield primarily, if not exclusively CD4+ cells, whereas dLyl cells or bone marrow precursors yield both CD4+ and CD8+ subsets. We have also evaluated the estrogen sensitivity of immature dLyl thymocytes, and the ability of limited pretreatment with estradiol to render the thymus of recipient animals transiently receptive for thymocyte precursors. Exogenous estradiol administration selectively depletes the large, subcapsular thymic blast population and permits thymic entry and some repopulation from donor-derived dLyl or bone marrow cells. An immature dLyl subpopulation has a selective growth requirement for a combination of IL1 plus IL2, but fails to further differentiate into other thymic subsets after the initial conversion of about 25% of dLyl cells to Ly-2+,L3T4+ cells, within 18-24 hrs. Finally, cells proliferating in response to IL1 and IL2 cannot repopulate the thymus of irradiated mice even when injected i.t., indicating that these cells are not representative of the freshly isolated dLyl precursor population.

PROJECT DESCRIPTION

PERSONNEL

Bonnie J. Mathieson	Head	LDS	LEI	NCI
Isabella Screpanti	Guest Researcher	LDS	LEI	NCI
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Llewellyn Mason	Microbiologist	LDS	LEI	NCI
John Wine	Bio. Lab. Tech.	CMIS	LEI	NCI
Kouji Matsushima	Visiting Associate	IS	LMI	NCI
Frances W. Ruscetti	Head	LS	LMI	NCI
Howard A. Young	Expert	IS	LMI	NCI

OBJECTIVES

The major objectives of this project are: 1) To study the normal intrathymic differentiation pathways in mice and to specifically compare the phenotype and functional potential of different thymic subsets by intravenous (i.v.) or intrathymic (i.t.) routes. 2) To determine the potential of thymocyte precursors that develop in mice pretreated with estradiol versus irradiation. 3) To develop enrichment and selection methods to obtain both immature and mature thymic subsets from fetal and adult organs and to determine the appropriate stages of cells for manipulation in vitro with biological response modifiers (BRM). 4) To develop methods to enhance the transfer and regeneration of thymus-derived cells in irradiated or immune deficient hosts. 5) To compare the transfer of thymic precursors with cells of similar phenotype from peripheral lymphoid organs. 6) To determine the anatomical location(s) of thymocyte progenitors and their descendants as they develop into more differentiated populations using adoptive transfer systems.

METHODS EMPLOYED

Cell suspensions of immature and mature thymocytes isolated by various methods have been used as potential sources for transfer i.v. and i.t. Immature adult thymocytes were obtained by lysis of the Ly-2+ cells with anti-Ly-2.2 monoclonal antibody (MoAb) plus complement (C) treatment, followed by selective cytotoxic elimination of the remaining mature L3T4+,Ly-1+,Ly-2- subset. This complete procedure yields less than 1% of normal adult thymocytes and must be obtained free of blood and peripheral lymphoid tissue to avoid contamination in the final population. Mature subsets of either Ly-2+ or L3T4+ cells were obtained by first eliminating all L3T4+ cells or Ly-2+ cells respectively by two cycles of MoAb + C treatment then selecting the Ly-2+ or L3T4+ cells by adherence to anti-Ig coated plates appropriate to the antibody being used. Double positive blasts were selected by anti-Ly-2 adherence followed by anti-L3T4 adherence before or after enrichment on Percoll density gradients.

Monitoring the cell subset purification and in vitro and in vivo differentiation in vivo was performed by flow cytometry analysis (FCA) in the BRMP Clinical Monitoring Unit, PRI. MoAbs and appropriate fluorescent detection reagents have been obtained through the laboratories that produced them, through the ATCC, or through commercial sources and used in these studies.

Selected immature thymocytes were cultured in 24 well plates or microtiter wells with natural purified and recombinant preparations of IL1, IL2, or IL3 and CSF, as well as the common T-cell mitogens. We also used Con A-induced spleen cell supernatants (CAS) for this investigation, and we have used selectively combined interleukins to promote proliferation. Finally, we have also studied the ability of these cells to develop when transferred back to the thymus in vivo.

Congenic animals used as recipients for thymocyte subsets were irradiated (700-1000 rads) or estradiol-treated (50 mg/day x 3 days). These animals have been monitored from 6-21 days in various experiments for donor and host phenotype after cell transfers i.v. or i.t. For localization studies, secondary, peroxidase-labelled antibodies were obtained commercially.

MAJOR FINDINGS

I. Isolation of Immature Thymocytes

A small subset of thymocytes with a phenotype which suggests that these cells are the equivalent of the thymic subcapsular cells has been previously reported. These cells have the following characteristics: they are dLyl cells by quantitative immunofluorescence; they express no detectable Ly-2 or L3T4. These cells have limited self renewal characteristics and are committed to the T cell lineage as demonstrated by a) their ability to repopulate all subsets of the thymocytes and b) their failure to yield multi-potential colony forming units in the spleen when transferred in vivo to irradiated animals. In addition the expression of IL2 receptor is seen on about 60% of these cells, and dLyl cells appear to be similar in phenotype to early fetal thymocytes that, by definition, are immature and can be obtained from mice at during fetal gestation from day 12 to 15.

The thymic subset of dLyl cells has no detectable NK activity and has only low levels of proliferative capacity without appropriate stimulation. However some dLyl cells can be converted in vitro to Ly-2+ cells within 24 hrs after which they die in culture. Repeated experiments indicate that a minority of the dLyl cells can be maintained without further differentiation in culture for several months with CAS without feeder cells. Under such conditions, these cells will develop cytoplasmic granules and develop the morphological appearance of NK cells, but these populations develop only low or negligible cytotoxic activity for NK targets. We have also stimulated dLyl cells with a panel of BRMs which can elicit proliferative responses. Only IL1 + IL2 or IL2 + PMA + ionomycin with or without Con A have been successful for this purpose.

Cell suspensions isolated from normal thymuses or in vitro stimulated thymus subsets have been injected into the thymus of irradiated and estradiol-treated mice. These experiments have allowed us to manipulate and assess the differentiation capacity of subpopulations whose potential for homing to the thymus is affected or limited by previous technical manipulations or the differentiation state of the cells.

The dLyl subset can be injected i.t. into irradiated hosts to repopulate the four major subsets within the thymus, and the sequence in which they appear is the same as that previously reported for i.v. transfers of dLyl cells. In

addition, the Ly-2+,L3T4+ blast population obtained from the adult thymus as well as the 16-day fetal thymus can be shown to give rise to mature cells after i.t. injection. Furthermore, both the mature L3T4+(Ly-2-) and Ly-2+(L3T4-) subsets were shown to proliferate and expand after i.t. injection in irradiated hosts. At the same time, not all immature thymocytes can be transferred i.t. Attempts to transfer early fetal thymocytes from day 13, 14, and 15 have been relatively unsuccessful, requiring cell numbers greater than one log higher than the adult dLyl cells for transfer. Whereas, both the Lyt2+,L3T4+ and the dLyl populations present at 16 days can yield donor-derived cells in irradiated animals. This indicates that the precursor capable of subset differentiation is very low in the fetal thymus. In addition, the dLyl population with a selected growth requirement for IL1 + IL2 which can be shown to proliferate in vitro cannot be transferred i.t. after several days in culture. This appears to reflect a loss not only of homing potential, which can be seen by i.v. injections, but also a loss of the ability to continue to differentiate or transfer when placed i.t. in irradiated hosts. We have concluded that the loss in potential of the ability to transfer and generate donor-derived thymocytes is due to an in vitro selection and expansion of a dLyl subset which is similar to the majority of early fetal thymocytes.

II. Attempts to Transfer Large Granular Lymphocytes (LGL) i.t.

There is extensive similarity in phenotype between fetal thymocytes, dLyl adult thymocyte precursors and LGL isolated from mouse spleen or liver (see accompanying report). Therefore, we have also attempted to transfer LGL by i.t. injection to determine whether these phenotypically similar cells had any potential as thymic precursors. In these studies, we have compared LGL isolated from mouse livers with dLyl cells and unfractionated bone marrow which contains the precursors for both NK cells and thymocytes. In these studies it is evident that dLyl and bone marrow cells successfully transfer i.t. donor-derived cells but LGL do not. By contrast LGL or bone marrow injected i.v. can transfer donor-derived LGL with NK activity.

III. A Selective Depletion in the dLyl Population by Estrogen-treatment of Animals

We have also examined the ability of different thymic populations to be transferred to congenic hosts treated with estrogen. Because estrogens administered to mice during prenatal and neonatal periods evoked impairment of thymus-derived immune functions, it was postulated that the estrogen receptors on low density thymocytes, which might include dLyl cells, would permit the transfer of donor-derived cells to specific niches within the thymus. We have tested this hypothesis and have determined that dLyl cells can indeed repopulate the thymus of estrogen-treated animals in a limited fashion. In contrast to the irradiation which has nonselective lympholytic effects, estrogen appears to deplete a selected population of subcapsular thymic cells in the adult animal. Thus, transferred dLyl cells appear to repopulate this subcapsular population in the estrogen-treated host. Surprisingly the early donor-derived repopulation in estrogen-treated animals with dLyl cells is approximately equivalent numerically to the donor-derived repopulation seen in irradiated animals. There is a difference, however, in a proportional repopulation because irradiated animals are more highly depleted at the time of irradiation than are estrogen-treated

animals. We are currently attempting to determine which subsets are generated after i.t. transfer to estrogen-treated congenic animals and whether non-congenic transfers will generate donor-derived cells.

IV. Localization of Injected Cells and Their Progeny

We have begun studies to monitor the thymic localization of donor-origin thymocytes with time following i.t. or i.v. transfer of thymocyte or bone marrow subsets. Preliminary experiments have indicated that donor-derived thymic cells from i.t. injected precursors can be detected from days 7 to 26 for dLyl transferred cells and from days 12 to 35 after bone marrow transfer. Furthermore, the immunoperoxidase assay is highly specific for detecting donor-vs.-host origin cells, and may permit us to analyze the sites of localization relative to other cells within the thymus that may be surrounding or associated with differentiating cells as they enter the thymus. In particular, we have begun to examine the association of IL-1 producing cells with the incoming (donor-derived) cells.

V. Studies of Oncogene Expression in Normal and Activated Subsets

In collaboration with several laboratories at the FCRF we have been analyzing different subsets of thymic lymphocytes for oncogenes which appear to have selective expression in lymphocytes. Studies with c-rel, have demonstrated that c-rel the cellular homolog of v-rel, which is the transforming gene of the leukemogenic reticuloendotheliosis retrovirus, plays a role later in stages of lymphocyte differentiation than has been reported for genes like c-myc or c-ets.

PUBLICATIONS

Brownell, E., Mathieson, B., Young, H.A., Keller, J., Ihle, J.N., and Rice, N.R.: Detection of c-rel-related transcripts in mouse hematopoietic tissues, fractionated lymphocyte populations, and cell lines. Mol. Cell. Biol. 7: 1304-1309, 1987.

Mathieson, B.J., and Sharrow, S.O.: Cell Surface Differentiation Antigens Expressed on Thymocytes and T Cells of the Mouse. In Miyasaka, M., and Trnka, Z. (Eds.): Differentiation Antigens in Lymphohemopoietic Tissues. New York, Marcel Dekker, Inc., 1987, pp. 65-151.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09300-01 LBP

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Microinjection Studies: Oncogenes' Role in Membrane Signal Transduction

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

PI:	H. Kung	Chief	LBP, NCI
Others:	S. Durum	Senior Staff Fellow	LMI, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (M. Smith): LBP, NCI (H. Kung); Clinical Immunology Section
PRI, NCI-FCRF (S. Kelley)

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

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

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

Microinjection of ras specific monoclonal antibody has been shown to block cellular DNA synthesis in normal and some transformed cell lines. This antibody also reverts the transformed morphology of ras transformed NIH 3T3 cells and induces these cells to flatten out taking on the appearance of normal NIH 3T3 fibroblasts. Fibroblast cells have been shown to be dependent upon a ras driven proliferation system. Lymphoid cell lines, macrophages and T cells, were serum starved and induced to proliferate by treatment with either IL-1, IL-2, or CSF. Microinjection of ras specific antibody had no effect upon factor induced cellular proliferation of these lymphoid lines, suggesting that IL-1, IL-2, or CSF induction of cellular growth does not depend upon a ras function.

A monoclonal antibody raised against the raf protein has recently been prepared that immunoprecipitates raf specific proteins at 74 and 44 Kd. When microinjected into NIH 3T3 cells, raf transformed NIH 3T3 cells or ras transformed NIH 3T3 cells the cells are blocked from entering the DNA synthesis phase of the cell cycle. This result suggests that ras and raf proteins are in the same membrane signal transduction pathway and that ras is upstream of the raf serine kinase in the proliferative pathway. The raf and ras antibodies are tools which we can utilize to study the biochemical interrelationships of the ras and raf proteins in cellular hormone induced membrane signal transduction.

Bacterial synthesized raf protein was partially purified and microinjected into serum depleted NIH 3T3 cells. Cells in the injected area of the cover slip were found to have entered the DNA synthesis phase of the cell cycle following raf injection, indicating that raf gene product is directly responsible for its transformation.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP	NCI
Scott Durum	Senior Staff Fellow	LMI	NCI

INTRODUCTION

Cancer cells isolated from tumors and transformed cells in culture are unrestricted in their growth potential. This common property of cancer cells is a result of genetic mutation that leads to an altered protein product capable of maintaining a continuous proliferative cell cycle. This proliferative signal in normal cells is activated by growth factor interaction with the cell membrane and is up regulated in transformed cells. The determination of the biochemical pathways involved in membrane signal transduction of various growth factors is the primary goal of our research.

OBJECTIVES

1. To elucidate the biochemical pathways involved in growth factor induced membrane signal transduction.

2. To study the biochemical interrelationships of the ras and raf proteins by microinjection of monoclonal antibodies.

3. To purify bacterially expressed raf protein and microinject the protein into serum starved fibroblasts.

METHODS

1. Microinjection of purified proteins or DNA's into living cells grown on coverslips.

2. Culture of cells in defined media to modulate cellular growth potential.

3. Protein purification and characterization.

4. Cellular immunofluorescence and autoradiography.

5. Molecular cloning techniques.

MAJOR FINDINGS

1. The product of the ras gene is required for a cell to enter the DNA synthesis phase of the cell cycle as demonstrated by the microinjection of ras directed monoclonal antibody. Antibody blocked the cells from entry into s-phase at late Go of the cell cycle.

2. Microinjection of ras monoclonal antibody will block progression of some retrovirus transformed NIH 3T3 cells (cells transformed by src, fes, fms, and

ras). Microinjection of ras monoclonal antibody does not block proliferation of raf and mos transformed cells. This observation suggests that many of the retroviral oncogenes are involved in similar or related biochemical pathways and that there is a molecular hierarchy extending from the outer surface of the cytoplasm to the nucleus of a cell.

3. Microinjection of a raf specific monoclonal antibody also blocks DNA synthesis of NIH 3T3 cells, raf transformed NIH 3T3 cells, and ras transformed NIH 3T3 cells. This observation demonstrates that the raf and ras oncogenes may be operating in the same biochemical pathway and that the ras protein is upstream from the raf protein. The membrane signal of a growth factor appears to go through ras before going onto raf. This metabolic pathway involves the interaction of ras and raf. Biochemical experiments are underway in the laboratory to address the interrelationships of these key regulatory proteins in vivo and in vitro.

4. Lymphoid cell lines traditionally have not been used for microinjection due to their nonadherence properties. Several factor dependent T cell lines have recently been identified that can be adhered to plastic coverslips for injection by concanavalin A agglutination. Only 5-10% of the cells enter S-phase of the cell cycle when held to coverslips by Con A. Upon addition of growth factor (IL-1, IL-2, or CSF) the number of cells entering DNA synthesis increases to 60-70%. If ras or raf specific monoclonal antibodies are microinjected into these T cells before factor addition no effect is observed upon cell entry into S-phase of the cell cycle. This result suggests that lymphoid cells may not require ras and raf for proliferation. This cell lineage may use an alternative progression biochemical pathway to mediate membrane signal transduction.

5. Bacterially expressed raf protein was partially purified by several column chromatography steps. Raf protein was microinjected into serum starved NIH 3T3 cells. Ten hours after injection the cells became morphologically transformed. Thymidine incorporation into DNA was used to measure entry of the cells into the S-phase of the cell cycle. It was found that injected raf protein induced cells to enter S-phase. This result demonstrates that the raf protein is sufficient to drive a cell into entering the DNA synthesis phase of the cell cycle and initiate progression.

PROPOSED COURSE

1. To complete microinjection of raf monoclonal antibody into various oncogene transformed cell lines in order to elucidate the functional relationships among various oncogenes.

2. To develop biochemical assays to study the interrelationships of the ras and raf proteins in normal cellular signal transduction.

3. To microinject other monoclonal antibodies raised against various oncogene proteins for growth modulating effects of fibroblast and lymphoid cells.

4. To examine other oncogene proteins of growth modulating effects of serum starved fibroblasts.

5. Similar studies will be performed in Xenopus oocyte system.

PUBLICATIONS

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Stacey, D. W., Watson, T., Kung, H. F., and Curran, T.: Microinjection of transforming ras protein induces c-fos expression. Mol. Cell. Biol. 7:523-527, 1987.

Deshpande, A. K., and Kung, H. F.: Insulin induction of Xenopus oocyte maturation is inhibited by monoclonal antibody against p21 ras proteins. Mol. Cell. Biol. 7:1285-1288, 1987.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09301-01 LBP

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Studies on the Biological Function of Human Ras Proteins

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

PI:	H. F. Kung	Chief, LBP	LBP, NCI
Others:	I. Calvert	Chemist, LBP	LBP, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (T. Kamata); LBP, NCI (H. Kung); Clinical Immunology Services
PRI, NCI-FCRF (S. Kelley) Clinical Immunology Services

LAB/BRANCH

Laboratory of Biochemical Physiology

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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 ras gene family code for a membrane protein p21 closely related to the G-protein family of a membrane signal transduction pathway. Studies were carried out to understand the role of ras proteins in phosphoinositide (PI) metabolic pathway triggered by growth factors.

PDGF stimulates PI turnover in normal rat kidney (NRK) cells and enhances hydrolysis of phosphoinositol monophosphate and bisphosphate (PIP2) in NRK cell membrane in the presence of GTPrs. Pertussis toxin does not inhibit the stimulatory effect of PDGF on P1P2 hydrolysis, suggesting that PDGF-stimulated phospholipase C activity is controlled by a G-protein which is different from Gi or Go. In contrast, the response to PDGF is completely lost in ras transformed cells, with the transformed cells showing a higher basal level of PI turnover than NRK cells. Using bacterially made normal or oncogenic T24 ras protein, we investigated the effect of ras proteins on inositol trisphosphate (IP3) production of NRK cell membrane in vitro. Normal ras protein increases the formation of IP3, whereas T24 ras protein does not have a significant effect. Moreover, normal ras protein and PDGF have additive effects on IP3 production. Our results indicate that normal ras protein may be coupled, indirectly or directly, with PDGF-stimulated phospholipase C activity and that oncogenic ras protein appears to be defective in this coupling. These findings are of considerable interest, implying the important regulatory role for ras proteins in cell transformation mechanisms.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP NCI
Ida Calvert	Chemist	LBP NCI

OBJECTIVES

The objectives of this project is to study the biological functions of normal or oncogenic ras protein in receptor-mediated membrane signal transduction, especially phosphoinositide metabolism.

MAJOR FINDINGS

It has long been postulated that ras proteins may play a regulatory role in membrane signal transduction by growth factors. Recent studies from several laboratories implicate that ras proteins may couple hormone receptors to phospholipase C which generates two second messengers, inositol trisphosphate (IP3) and diacylglycerol. However, these works are based on indirect in vivo observations. Therefore, we decided to conduct direct biochemical studies on this issue.

I. Involvement of a G-protein in PDGF-Stimulated Phospholipase C Activity.

First, we examined the effect of growth factors on phosphoinositide (PI) turnover in untransformed and ras transformed cells. Our data showed that PDGF stimulates PI turnover in NRK or NIH 3T3 cells, while insulin or EGF has little or no effect. PDGF enhances hydrolysis of phosphatidyl inositol monophosphate (PIP) and bisphosphate (PIP2) in normal NRK cell membrane in the presence of GTPrS, but Harvey- or Kirsten murine sarcoma virus transformed cells, completely lack the response to PDGF. The basal level of PI turnover is higher in ras transformed cells than in untransformed cells. Pretreatment of NRK cells with pertussis toxin does not suppress the stimulatory effect of PDGF on PIP2 hydrolysis. Taken together, these results imply that PDGF-stimulated phospholipase C activity of NRK cells is regulated by a guanine nucleotide binding protein which is insensitive to pertussis toxin and that ras transformation suppresses the response of PI metabolism to PDGF.

II. Effect of E. coli ras Proteins on Phospholipase C.

Next we attempted to clarify whether the purified ras protein could couple the receptor to the phospholipase C-mediated PI hydrolysis in a cell-free membrane system. For this purpose, we used the in vitro reconstitution system with purified human ras proteins expressed in E. coli and 32P-labeled NRK cell membrane. We demonstrated that normal ras protein stimulates by three fold the formation of IP3 in the presence of GTPrs, whereas oncogenic ras protein does not have a significant effect. Furthermore, the stimulatory effect of normal ras protein on IP3 production is additive to that of PDGF. This raises two possibilities: (1) normal ras protein may, directly or indirectly, affect PDGF-stimulated phospholipase C activity, and (2) the actions of normal ras protein and PDGF on phospholipase C may involve a separate route. In any case, oncogenic ras

protein appears to be defective in the coupling with phospholipase C, and the lack of PDGF-response by ras transformation may correlate with this defectiveness of oncogenic ras protein. Our results would for the first time provide an insight into the functional difference between normal and oncogenic ras protein and argue against the hypothesis that oncogenic ras protein activates directly phospholipase C activity.

FUTURE PLANS

Unlike the mammalian ras proteins, ras proteins produced in a bacterial expression system is not lipidated. The possibility that an "unprocessed" form of ras protein may behave differently from "processed" ras proteins remains to be investigated, for example, the lipidated ras proteins may interact more efficiently with membrane components. In order to test this possibility, we will purify processed ras proteins from mammalian sources (e.g. bovine brain tissue which is enriched in normal ras protein) and conduct a similar in vitro reconstitution experiment. In another experiment, we will further explore the possible interactions between ras proteins, purified phospholipase C, and growth factor receptors (for PDGF, insulin and EGF). Ultimately, these studies will give us information about the precise mechanism of ras-regulated signal transduction.

PUBLICATIONS

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Willumsen, B. M., Papageorge, A. G., Kung, H. F., Bekesi, E., Robins, T., Johnsen, M., Vass, W. C., and Lowy, D. R.: Mutational analysis of a ras catalytic domain. Mol. Cell. Biol. 6:2646-2654, 1986.

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Deshpande, A. K., and Kung, H. F.: Insulin induction of Xenopus oocyte maturation is inhibited by monoclonal antibody against p21 ras proteins. Mol. Cell. Biol. 7:1285-1288, 1987.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09302-01 LBP

PERIOD COVERED
October 1, 1986 - September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
G-Proteins and Signal Transduction

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

P.I.:	H. F. Kung	Chief	LBP, NCI
Others:	J. Clark	Senior Staff Fellow	BTB, NCI
	J. Moss	Deputy Chief	LBI, NCI
	M. Vaughan	Chief	LBI, NCI

COOPERATING UNITS *(if any)*
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3.0	2.5	0.5

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

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

Guanyl nucleotide binding proteins (G-protein) are critical components of several regulatory pathways in animal cells, specifically involved in signal transduction. Recent evidence suggests the involvement of G-proteins in the regulation of various BRM's and alterations in their function may contribute to the pathogenesis of disease. Of these G-proteins Go is present in large quantities in critical organs like vertebrate brain and heart. Its function is not known and hence these studies are aimed at understanding the role of this protein in various signal transduction pathways and its role in various malignancies associated with brain. A bovine retinal cDNA clone encoding the complete alpha subunit of Go, was isolated and sequenced, which encodes for 354a.as polypeptide (Mr 39,900). Comparison of this G-protein with other G-proteins has revealed good homology at important critical sites like GTP binding, GTP hydrolysis, and at sites ADP ribosylated by cholera toxin and pertussis toxins. Using the cDNA clone as a probe, genomic clones coding for Go alpha were obtained and characterized. Three clones encompassing 20Kb, coding for the 5' part of the coding region were characterized, and the first 3 exons were sequenced. Also, using the bovine Go alpha cDNA clone as a probe, we have obtained cDNA clones from a human brain library, clones from human brain library-Alzheimer's tissue and from a Neuroblastoma library. The cDNA clones from the normal human library are sequenced and found to have strong homology to the bovine cDNA clone.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP	NCI
Jeff Clark	Senior Staff Fellow	BTB	NCI
Joel Moss	Deputy Chief	LCM	NHLBI
Martha Vaughan	Chief	LCM	NHLBI

INTRODUCTION

Guanyl nucleotide binding proteins (G-protein) are critical components of several regulatory pathways in animal cells, specifically involved in signal transduction. Recent evidence suggests that G-proteins may play a role in mediating the signal transduction of various BRM's, and alterations in their function may contribute to the pathogenesis of disease. (Evans et.al., 1987, Science 325,166) (Growler, et.al., 1987,Nature, 327,229) The product of the ras oncogene is also a G-protein, and mutations of this gene appear to be important in oncogenesis being found at increased frequency in a variety of human malignancies. G-proteins are a multi-gene family with alpha, beta and gamma subunits. Beta and gamma subunits of G-proteins appear to be similar but alpha subunits are unique to each G-protein.

OBJECTIVES

Go is present in large quantities in critical organs, especially vertebrate brain and heart. Its function is not known and hence these studies are aimed at understanding the role of this protein in various signal transduction pathways and its possible role in various neuronal malignancies.

METHODOLOGY

1. DNA extraction and restriction enzyme analysis.

2. Southern blotting, Northern blotting and hybridization.

3. Making genomic libraries, cDNA libraries and screening for recombinant clones.

4. Detailed characterization of clones.

5. Nucleotide sequence analysis.

MAJOR FINDINGS

A bovine retinal cDNA clone encoding the complete alpha subunit of Go, was isolated and sequenced, which encodes for 354a.as polypeptide (Mr 39,900). Comparison of this G-protein with other G-proteins has revealed good homology at important functional sites (GTP binding, GTP hydrolysis, and at sites ADP ribosylated by cholera toxin and pertussis toxins). Studies are underway to study the expression of this gene in bacteria and mammalian cells.

Using the cDNA clone as probe, genomic clones coding for Go alpha were obtained and characterized. Three clones encompassing 20Kb, coding for the 5' part of the coding region were characterized, and the first 3 exons were sequenced. Using a 3' specific probe, five more clones were obtained, and the detailed characteri-zation of these clones are underway.

Also, using the bovine Go alpha cDNA clone as a probe, we have obtained cDNA clones from a human brain library, clones from human brain library-Alzheimer's tissue and from a Neuroblastoma library. The cDNA clones from the normal human library are sequenced and found to have a polypeptide of 305 amino acids, but lacking the 5' region of 49 amino acids. Presently, we are screening the library again with a 5' specific probe to obtain the full length clone. Detailed characterization of the cDNA clones from the Alzheimer's tissue and from the Neuroblastoma library are in progress.

FUTURE PLANS

Studies are planned to express these cDNA clones in mammalian cells and in bacteria and to study the effect of in vitro mutagenesis of the human cDNA clones on their function. Better understanding of the function of G-proteins and their role in the transduction of various external signals should provide insight into the role of at least a subset of these proteins (the ras genes) in oncogenesis.

The genomic clone will be used in studying the regulatory elements of the gene and to look at potential alternative splicing events which have been reported for human Gs alpha clones.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09312-01 LBP

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Mechanisms of Action of the Interferons

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

PI:	H. F. Kung	Chief	LBP, NCI
Others:	G. Princler	Chemist	LBP, NCI
	M. Sparks	Guest Researcher	LMI, NCI
	F. Ruscetti	Microbiologist	LMI, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (C. Faltynek); LBP, NCI (H. Kung); Clinical Immunology Services, PRI, NCI-FCRF (R. Overton, L. Finch)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.0	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.)*

After binding to specific cell surface receptors, interferon-alpha (IFN-alpha), like other polypeptide hormones and growth factors, is internalized by the cells via receptor-mediated endocytosis. We have been studying the role for internalization of IFN-alpha in responses to IFN-alpha and have found that concanavalin A (Con A) effectively inhibits both the internalization of IFN-alpha and the induction of 2',5' oligo (A) synthetase activity in IFN-treated cells. Similar dose response curves were obtained for the effect of Con A to inhibit IFN internalization and 2',5' oligo (A) synthetase induction. At least part of the effect of Con A to inhibit the induction of 2',5' oligo (A) synthetase activity was attributable to inhibition of induction of 2',5', oligo (A) synthetase mRNA. The effect of Con A to inhibit protein mobility in the plasma membrane and the correlation between the Con A-mediated inhibition of 2',5' oligo (A) synthetase induction and inhibition of IFN-alpha internalization supports the hypothesis that internalization of IFN receptor complexes and/or receptor mobility may be necessary for some of the responses to IFN-alpha.

In studies on the antiproliferative action of IFN-alpha, we have found that glucocorticoids and IFN-alpha synergistically inhibit the growth of some B lymphoblastoid and B lymphoma cell lines. These findings may have clinical relevance in the treatment of certain lymphoid malignancies sensitive to IFN-alpha. In studies on the possible role that the IFN's may play in treatment of AIDS, we have found that IFN-gamma treatment of myelomonocytic cell lines decreases expression of the T4 cell surface protein, which has been implicated to be a receptor for the AIDS virus, HIV-1. This decrease in T4 expression is not due to a generalized loss of cell surface antigens, since several other antigens are either unchanged or increased in IFN-gamma treated cells.

PROJECT DESCRIPTION

PERSONNEL

Hsiang-fu Kung	Chief	LBP	NCI
Gerald Princler	Chemist	LBP	NCI
Maria Birchenall-Sparks	Guest Researcher	LMI	NCI
Frank W. Ruscetti	Senior Investigator	LMI	NCI

OBJECTIVES

The IFN's are a family of proteins with potent antiviral, antiproliferative and immunomodulatory activities. Although, the mechanisms in the action of the IFN's as antiviral agents are fairly well understood, very little is known about the biochemical and cell biological events which lead to the antiproliferative and immunodulatory activities of the IFN's. The objective of this project is to elucidate such events. Specific objectives include studies on the role for IFN internalization and IFN receptor modulation in responses to IFN, studies on the antiproliferative activity of IFN alone, and combined with other biologically active agents using cultured cell lines, and studies of the IFN's as differentiation agents and modulators of cell surface antigen expression.

MAJOR FINDINGS

As with other polypeptide hormones and growth factors, after binding to specific cell surface receptors, IFN-alpha along with its receptor is internalized by the cells. However, the physiological significance of the internalization of IFN is not known. We have found that the lectin Con A, which does not inhibit the binding of 125I-rIFN-alpha A, effectively inhibits both the internalization of 125I-rIFN-alpha A and the induction of 2',5' oligo (A) synthetase by rIFN-alpha A in the B lymphoblastoid cell line Daudi. The inhibition of IFN-induced 2',5' oligo (A) synthetase activity by Con A was due neither to direct inhibition of the enzymatic activity, to induction of an inhibitor of 2',5' oligo (A) synthetase activity, nor to a generalized inhibition of protein or RNA synthesis. Instead, when present during the incubation with rIFN-alpha A, Con A inhibited the induction of 2',5' oligo (A) synthetase activity at least in part by inhibiting the induction of 2',5' oligo (A) synthetase mRNA. The dose response curves were similar for the effect of Con A to inhibit 125IrIFN-alpha A internalization and 2',5' oligo (A) synthetase induction by rIFN-alpha A.

We also found that for a maximal induction of 2',5' oligo (A) synthetase, rIFN-alpha A needs to occupy receptors for longer than 2 hours, suggesting that rapid transient signals generated at the plasma membrane by the binding of rIFN-alpha A to its cell surface receptor may not be sufficient to account for maximal responses to rIFN-alpha A. The correlation between the Con A mediated inhibition of 125I-rIFN-alpha A internalization and 2',5' oligo (A) synthetase induction suggests that internalization of rIFN-alpha A may play a role in the responses to rIFN-alpha A. However, Con A also inhibits protein mobility in the plasma membrane. It is possible that Con A is preventing either required homologous interactions between IFN receptors or heterologous interactions

between IFN receptors and signal transducing proteins in the plasma membrane. Studies are in progress to address these questions.

It is well known that IFN-alpha inhibits the growth of many types of cells in vitro and has efficacy in vivo for the treatment of some malignancies, especially certain lymphoproliferative diseases. Glucocorticoids also have a suppressive action on lymphoid tissue. We have recently found that rIFN-alpha A and gluco-corticoids act synergistically to inhibit the growth of some B lymphoblastoid and lymphoma cell lines in vitro. These findings may have clinical relevance in the treatment of lymphoid malignancies.

There is considerable evidence that the human cell surface protein T4 is not only an important accessory molecule in the activation of MHC Class II-restricted T cells, but is also the receptor for the AIDS virus HIV-1 on lymphoid, myeloid and brain cells. We have been studying the effect of lymphokines and cytokines on T4 expression. We have found that treatment of the promonocytic cell line U937 with rIFN-gamma decreases the expression of the T4 antigen by 50% as measured by FACS analysis. The decrease in T4 expression was dependent on the concentration of rIFN-gamma, with maximal effects occurring at 20-200 u/ml. The decrease was due to actual loss of the T4 molecule from the cell surface rather than masking of a particular epitope, since similar results are obtained with the OKT4 and OKT4a antibodies. The maximal decrease occurred after 24 hours treat-ment with rIFN-gamma; subsequently the level of T4 increased. The effect of rIFN-gamma to decrease T4 expression was not due to a generalized loss of cell surface antigens, since the levels of Fc receptor, C3bi receptor, and HLE were either increased or were unchanged after treatment with rIFN-gamma. GM-CSF and, to a lesser extent, rIFN-alpha, but not rIL-2, also decreased the level of T4, but the decrease was less than observed with rIFN-gamma and was more variable. Treatment of the promyelocytic leukemia cell line HL-60 with rIFN-gamma for 24 hours also decreased cell surface T4 expression, although higher concentrations of rIFN-gamma were required with HL-60 than with U937 cells for maximal decrease. We are currently studying whether the decreased expression of the T4 antigen is related to the state of cellular differentiation and whether the rIFN-gamma induced loss of cell surface T4 is due to increased internalization and degra-dation or decreased synthesis of T4.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09278-04 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Biodistribution and Trafficking of Normal and Activated Blood Monocytes

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

PI:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
Others:	J. Beman	Nurse Specialist	CRB, NCI
	P. Miller	Biologist	OAD, NCI
	R. G. Steis	Chief	CRB, NCI

COOPERATING UNITS *(if any)*

Nuclear Medicine, Clinical Center, NIH (Andrew Keenan); Frederick Memorial Hospital (Margie Farrell); Genetech, San Francisco, CA (Steven Sherwin).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This research study is designed to assess the trafficking pattern of autologous monocytes when given to humans intravenously. Monocytes will be removed from normal volunteers or from cancer patients by cytapheresis and purified by counter-current centrifugal centrification. The purified monocytes will either be left at 4° (normal monocytes) for 4 hours or be activated by exposure to recombinant human gamma interferon. The cells will be labeled with Indium-111 and infused intravenously back into the normal donor or patient, respectively. Normal donors or cancer patients will first be given an intravenous infusion of autologous normal monocytes; if no untoward reactions are noted, the donors or patients may receive infusions of autologous gamma interferon-activated monocytes four weeks later. Frequent blood samples will be withdrawn from the patient or donor over the ensuing 96 hours to determine the rate at which the infused cells equilibrate with the marginating pool of blood monocytes (if one exists), and to determine the rate at which these cells emigrate from the blood stream to enter the reticuloendothelial system and other tissues. In addition, multiple regional scintagraphs will be obtained to determine if these labeled monocytes preferentially localize to any one organ system. This information will further our understanding of the normal physiology of activated and unactivated monocytes and, in addition, hopefully will allow us to more rationally design adoptive immunotherapy trials in which purified autologous cytotoxic blood monocytes will be given to cancer patients intravenously.

PROJECT DESCRIPTION

PERSONNEL

John W. Smith II	Senior Staff Fellow	CRB NCI
JoAnn Beman	Nurse Specialist	CRB NCI
Paul Miller	Biologist	OAD NCI
Ronald G. Steis	Chief	CRB NCI

OBJECTIVES

The objectives of this study are:

1. To determine the toxicity in cancer patients and normal donors of intravenously infused autologous monocytes.

2. To study the biodistribution and trafficking of intravenously infused autologous monocytes.

3. To evaluate the possible antitumor effects of autologous monocytes in cancer patients.

METHODS EMPLOYED

Normal volunteers or patients will be admitted to the Biological Response Modifiers Program Cytapheresis Unit for leukapheresis. There will be minimal anticoagulation of the normal donor's or patient's blood using acid citrate dextrose and heparin. White cells will be collected for exactly 2 hours at which time approximately 5×10^9 mononuclear cells will have been collected using a Celltrifuge 2 cell separator or CS 3000 cell separator. The donors will then be admitted to the Biological Response Modifiers Program inpatient unit. The leukocytes collected by leukopheresis will then be subdivided into mononuclear cells using hypaque-ficoll gradients according to standard procedures. These leukocytes will then be washed 3X in RPMI 1640 media (sterile and endotoxin-free) and entered into an elutriator centrifuge in medium containing RPMI 1640, and clinical grade human albumin (Cutter Laboratories). The mononulcear leukocytes will be entered into the elutriator in a laminar flow hood and the monocytes will also be collected out of the elutriator centrifuge in a similar hood. The purified monocytes will then be incubated at 4° for 4 hours or exposed to .5 microgram/ml of recombinant gamma interferon for 4 hours at 37°. Following the 4 hour incubation period, the cells will be washed twice in RPMI 1640, and 5×10^8 monocytes will be labeled with 0.25 millicuries of ^{111}indium oxine, according to the technique of Lotze et al., previously employed in our earlier EVLA studies. The ^{111}indium labeled monocytes will be washed twice in PRMI 1640 and resuspended in phosphate buffered saline + 10% albumin and infused into the patient or normal donor intravenously over 5 minutes. The total body maximum radiation dosage estimate for the ^{111}indium labeled monocytes administered intravenously has been calculated to be .710 rads/millicurie. Since only 0.1 of a millicurie will be injected per patient or normal donor, a maximum radiation exposure estimate per procedure is .071 rads of total body exposure. The critical organ is expected to be the

spleen, 0.249 rads per study. Infused cells (in the medium in which they are suspended) will be routinely tested for endotoxins, bacteria and fungi; in addition, their purity, viability, and tumoricidal function will be monitored.

In order to determine the biodistribution of ^{111}indium labeled monocytes infused intravenously, whole body images and multiple regional scintiphotos of the torso will be obtained on a daily basis throughout the five days of the study and acquired on computer for later analysis. In addition, blood samples will be obtained every 10 minutes for the first 2 hours of the study, on an hourly basis for the next 16 hours of this study, and on a every 12-hour basis for the duration of the study.

MAJOR FINDINGS

Five cancer patients have completed treatment with this study using both activated and non-activated autologous monocytes. No toxicity was seen with the nonactivated monocytes. Two patients receiving activated monocytes had minor toxicity (low grade fever in one; fever, fatigue, chills, and myalgias in the other) which was easily controlled symptomatically and resolved promptly by the end of therapy. Images revealed no uptake of monocytes at sites of tumor deposits. No antitumor responses were seen in any patient. Four normal donors have now received nonactiviated autologous monocytes labelled with ^{111}Indium. No toxicity was observed. In both the cancer patients and in normal donors, most of the monocytes remained in the peripheral circulation but for a very short period of time. The number of counts per milliliter in the peripheral blood subsequently fell dramatically only to rise at a later time point. This presumably reflects trafficking of these monocytes to extravascular spaces followed by slow return of the monocytes over time.

SIGNIFICANCE

For the last five years, several investigators in the BRMP have been characterizing the ability of highly purified human monocytes to kill cancer cells in vitro. It is now possible to routinely isolate up to 10^9 highly purified human monocytes from normal volunteers or cancer patients by the techniques outlined above and monitor their ability to kill cancer targets in vitro. Baseline cytotoxic capabilities of these monocytes can be routinely boosted by in vitro incubation with gamma interferon. The question that is being addressed in this particular protocol centers around the general applicability of adoptively transferred cytotoxic monocytes in the treatment of cancer. Since most patients with metastatic cancer do not have disease limited to one anatomic space, it would be most reasonable to attempt to approach these patients with adoptively transferred monocytes through intravenous infusions. It appears from the preliminary results of the current study that either activated or unactivated monocytes do not home to sites of tumor. Subsequent studies might take advantage of this observation and try to target monocytes by incubating them not only with gamma interferon but also with appropriate monoclonal antibodies. One goal of the present study is to determine the biodistribution of activated and unactivated monocytes to determine if there is a need for alternative methods to attempt to make them home to sites of disease. It is felt that the present study is warranted because of human and animal in vitro data suggesting that monocytes when properly activated can have

direct tumoricidal activity in vitro and because of animal in vivo experiments suggesting that activated macrophages can check the spread of or eliminate established tumors. The patient population in normal donors described in this protocol are reasonable for testing the trafficking of intravenously infused monocytes since there is no direct information as to the behavior of these cells (either in their baseline or activated stage) when infused intravenously. If a mechanism can be found whereby activated monocytes can be targeted to tumor sites, protocols will be developed for the treatment of widely disseminated metastatic cancer with such activated cells.

PROPOSED COURSE

As detailed in the Methods Employed section.

PUBLICATIONS

Stevenson, H.C., Lacerna, L.V., and Sugarbaker P.: Adoptive Immunotherapy: Activated human blood monocytes. Clin. Res. 35(3): 528A, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09279-04 CRB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phase I Trials of Interleukin-2 in Patients with Cancer

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

PI:	J. Clark	Senior Staff Fellow	CRB, NCI
Others:	R. G. Steis	Chief	CRB, NCI
	R. L. Miller	Senior Staff Fellow	CRB, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*
Frederick Memorial Hospital; PRI, NCI-FCRF (Walter Urba and Edward Crum).

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

This trial was designed to evaluate the immunomodulatory effects and toxic effects of interleukin-2 in patients with cancer. The interleukin-2 was given subcutaneously, intramuscularly, or by slow intravenous infusion for 24 hours in single escalating doses given once per week. The pharmacokinetics of IL-2 and the effects of the IL-2 on various immune parameters were monitored serially. Each individual patient then received daily intramuscularly injections of interleukin-2 for 5 consecutive days for 3 consecutive weeks. The dose of interleukin-2 used during this 3-week period was that dose which optimally augmented the immune function during the initial dose-escalation phase of the study.

Thirteen patients are evaluable for response and toxicity on this trial. No tumor responses were seen. Single doses of interleukin-2 were incapable of significantly augmenting immune function even with doses up to 3 times 10 to the 7th units per meter squared. When patients received daily injections of interleukin-2, significant alterations in immune function were observed. There were persisting and progressive elevations in the soluble IL-2 receptor level, natural killer cell activity in the peripheral blood, and the appearance of Leu-19 positive cells in the peripheral blood. These changes were progressive over time but returned to baseline within 4 weeks. Based upon these favorable effects on the immune system, interleukin-2 is being administered in a new trial at moderate dosages for prolonged periods of time.

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

PROJECT DESCRIPTION

PERSONNEL

Jeffrey Clark	Senior Staff Fellow	CRB	NCI
Ronald Steis	Chief	CRB	NCI
Robin Miller	Senior Staff Fellow	CRB	NIC
Dan L. Longo	Associate Director	OAD	NCI

OBJECTIVES

The objectives of this study are:

1. To determine the toxicity in cancer patients of recombinant interleukin-2 (IL-2) when given subcutaneously, intramuscularly or by slow intravenous injection in escalating doses.

2. To study the pharmacokinetics and immunomodulatory properties of interleukin-2 when given by these three routes and in escalating doses.

3. To evaluate the possible antitumor effects of interleukin-2 in cancer patients.

METHODS EMPLOYED

Recombinant interleukin-2 is produced by standard genetic engineering technology and expanded in E. coli. We have obtained an interleukin-2 preparation from the Hoffmann LaRoche Corp. which is felt to have the lowest contamination levels of endotoxins and the highest specific activity of the various interleukin-2 preparations that we have tested to date. This clinical grade material has passed FDA inspection including pyrogenicity testing. The patients eligible for this protocol are cancer patients with histologically confirmed diagnosis for which there is no standard or effacious therapy. T-cell malignancies are excluded (because of the possibility the interleukin-2 might stimulate the growth of these malignant cells). The patients must be between the ages of 18 and 70 and have a life expectancy of at least 3 months and a performance status greater than 60% of the Karnovsky scale. In addition to giving informed consent these patients must have normal hemalogic, renal, and liver function. Also, the omission of previous chemotherapy, radiation therapy, or biological response modifier therapy for 4 weeks prior to entry onto the protocol is required. Two basic study plans (Schedule A and Schedule B) for interleukin-2 have been identified. On Schedule A, patients are randomized to one of three infusion routes (i.v., i.m., or subcut) and patients receive weekly escalating doses of interleukin-2 from 10^3 to 10^7 units per meter square. In addition to monitoring toxicity and defining the maximal tolerated dose (MTD), the optimal immunomodulating dose (OID) is defined. Following a three week wash out period, each patient will receive the OID amount of interleukin-2 i.m. on a daily basis for three weeks (Schedule B). The immunomodulatory testing that is performed throughout the study includes assessment of peripheral blood spontaneous lymphocyte proliferation in vitro and the proliferative responses of these cells to lectins and a mixed leukocyte response (MLC) stimulus. In addition, T-cell subsets, B cells and natural

killer cells and monocytes are enumerated by fluorescence activated cell sorter analysis. Also, natural killer cell activity, monocytemediated cytostasis and studies of soluble interleukin-2 receptor levels are performed.

Patients on Schedule B are randomized to receive OID amounts of interleukin-2 on a daily basis for three weeks. Patients demonstrating at least stable disease during the course of interleukin-2 treatment at the end of three weeks are eligible to continue receiving the agent according to their Schedule B protocol. If stable disease continues after subsequent courses of interleukin-2 the patient will be continued on the study at the discretion of the principal investigator. If grade 4 toxicity is observed, interleukin-2 will be discontinued. Similarly, patients demonstrating a growth in their tumor over the time of treatment will be taken off the study.

MAJOR FINDINGS

So far fourteen patients have been treated of whom thirteen are evaluable for response and toxicity. One patient was removed after two single doses of interleukin-2 because of rapid tumor progression. No tumor responses have been seen to date. Toxicity has been observed at doses of $10^6/m^2$ and above and were considered mild. These toxicities consisted of diarrhea, fatigue, rash, pruritus, and fever which were easily controlled symptomatically and promptly resolved after therapy was stopped. At $10^7/m^2$, patients developed fluid retention, moderate hypotension, decreased urine output, and anxiety. There was one death in a patient with malignant melanoma at the $10^7/m^2$ dose level. Autopsy in this patient failed to reveal an obvious cause for death. Clinically, the patient was stable during the course of his interleukin-2 treatments. He had unsuspected brain metastasis found at autopsy and in addition was found to have a very small septic pulmonary embolus possibly related to an in-dwelling central venous catheter. However, there was no significant pulmonary embolus, and the small septic embolus that was found was not deemed to be large enough to have caused hemodynamic compromise. Because this patient was receiving interleukin-2 at the time of his death, his death is being called a treatment-related death though the mechanism by which interleukin-2 caused this is certainly not clear at this point.

As mentioned, very few changes in immune parameters were observed during the course of the administration of the single escalating doses of interleukin-2. However, significant and persisting changes in immune parameters were observed when patients received daily injections of interleukin-2.

SIGNIFICANCE

One of the major goals of the Biological Response Modifiers Program is to develop new insights into understanding the role of the immune system in the immunosurveillance or rejection of clinical malignancy. Administration of interleukin-2 along with LAK cells has been associated with significant antitumor effects in selected groups of patients. This process, however, is time consuming, expensive, and very laboratory and physician intensive. One way to potentially overcome these drawbacks of interleukin-2 and LAK cell therapy is to attempt to bring about the generation of lymphokine activated killer cells in vivo by the administration of interleukin-2 alone. In

attempting to do this, it would be necessary to determine the effects of single and then multiple doses of interleukin-2 on the immune system. With the information we have obtained on this study so far it appears that single, even very high doses of interleukin-2 are incapable of significantly augmenting immune function. Changes in immune function that might correlate with antitumor effects have been observed on this trial only when interleukin-2 is given on a continuous basis. The doses utilized have been at most moderately high doses, and thus, it might be possible with continued administration of moderate doses of interleukin-2 to bring about the generation of in vivo LAK activity with hopefully subsequent favorable clinical responses. Our data would suggest that high and very poorly tolerated doses of interleukin-2 might not be necessary to achieve this end. We feel, therefore, that the findings of this current trial have direct applicability to the design of future trials. In fact, a trial using interleukin-2 given twice per week in moderately high doses has now been designed and will soon be starting in the intramural program at the BRMP.

PROPOSED COURSE

At the moment, only one patient remains on treatment on this study receiving 1 million u/m^2 intramuscularly twice per week. She is being monitored for toxicity (very little toxicity has been observed so far) and immune function is being evaluated. So far her disease (nodular mixed lymphoma), has remained stable.

PUBLICATIONS

None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09291-02 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Alternating 2'-dCF in Recombinant Leukocyte A INF in Hairy Cell Leukemia

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

PI:	R. G. Steis	Chief	CRB, NCI
Others:	A. Martin	Staff Fellow	DEB, NCI
	S. Nerenstone	Staff Fellow	DEB, NCI
	M. J. Hawkins	Head, Biological Eval. Sect.	CTEP, NCI
	R. Simon	Chief	BR, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, Maryland 21701 (W. Urba).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Hairy cell leukemia has been found to be very responsive to recombinant alpha interferon therapy. Interferon alone, however, is incapable of curing people with this disease though in the vast majority of patients, the peripheral blood counts can be normalized. 2' deoxycoformycin by itself is an effective modality of therapy in this disease and is capable of producing substantial responses in patients resistant to prior alpha interferon therapy. This study is a pilot study to determine the toxicities and antitumor effects of the combination of deoxycoformycin and interferon. Fifteen patients have been admitted to the study, and 13 are evaluable for response, the other 2 being too early. There have been 10 partial responses and 3 complete responses. This rate of complete remission appears to be lower than that published in trials using deoxycoformycin as a single agent. However, those studies have used a single unilateral iliac crest bone marrow biopsy as part of the basis for the definition of a complete response. Our definition of complete remission requires that 2 sets of bilateral iliac crest bone marrow biopsies be negative for hairy cells. Our apparent lowered response rate, thus, might be a reflection of our more stringent criteria for complete remission. Bone marrow aspirates and biopsies from our trial and from extramural trials utilizing deoxycoformycin alone will be evaluated by a single pathologist to attempt to address this discrepancy. However, even if our complete response rate doubles as a result of this external review, it appears that our complete remission rate would be no better than in patients receiving dCF alone. Therefore, this study is closed to further patient accrual.

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

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Allison Martin	Staff Fellow	DEB	NCI
Stacy Nerenstone	Staff Fellow	DEB	NCI
Michael J. Hawkins	Head, Biological Eval. Sect.	CTEP	NCI
Richard Simon	Chief	BR	NCI
Dan L. Longo	Associate Director	OAD	NCI

OBJECTIVES

1. To determine the qualitative and quantitative toxicities of 2'-deocycoformycin and interferon alpha 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 alternations in immune function as a result of treatment with alternating dCF and alpha interferon.

4. To determine the biochemical consequences of administering dCF with a biological response modifier.

METHODS EMPLOYED

Patients to be considered eligible for this study must have morphologically identifiable hairy cells in the peripheral blood, bone marrow or tissue biopsies including a positive stain for tartrate-resistant acid phosphatase. They must meet minimal criteria for peripheral blood counts prior to starting therapy. Hemoglobin must be 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 3 months prior to study entry. Prior therapy is allowed for patients entering this study, but no patients must have received prior alpha interferon or deoxycoformycin. Patients are evaluated in the outpatient clinic of the Clinical Research Branch and then are hydrated and given 4 mg/m^2 of deoxycoformycin intravenously as a one-half hour infusion. After infusion of the dCF, a further liter of fluid is administered to insure adequate hydration. Patients are serially evaluated for myelosuppression and receive weekly injections at this dose of deoxycoformycin for three consecutive weeks. On week 4 of the study no therapy is given, and in weeks 5 through 8 daily subcutaneous injections of recombinant alpha interferon at a dose of 3 million units/m^2 is administered. This two-month cycle of deoxycoformycin alternating with interferon is repeated for a minimum of 7 cycles. Initial response is evaluated by serial evaluations of peripheral blood counts and by bilateral iliac crest bone marrow biopsies performed at the 6-month time point. Bilateral iliac

crest bone marrow biopsies are repeated at the time the patient ends therapy at the completion of the seventh cycle of therapy. Other bone marrow biopsies may be repeated between the sixth and fourteenth month of therapy as clinically indicated. For patients clearly responding well to therapy and tolerating the therapy reasonably well, some of the deoxcyformycin injections are permitted to be administered by the local physician. These local physicians, however, must agree to follow the protocol exactly as written.

MAJOR FINDINGS

As mentioned, 15 patients have been admitted to this trial, of whom 13 are evaluable for response. One patient has just started therapy and is not evaluable for response. All patients had progressive hairy cell leukemia and one or more cytopenias. The major toxicities related to deoxycoformycin so far have included myelosuppression during the first or second course of dCF administration and nausea and vomiting developing between 12 and 24 hours after drug administration. So far, 4 of the 15 patients admitted to this study have been admitted to the hospital because of fever and granulocytopenia.

Of 13 patients evaluable for response so far (2 are too early for response), there have been 3 complete remissions and 10 partial remissions. Complete remission in our study is said to be present when 2 consecutive sets of bilateral iliac crest bone marrow biopsies do not contain any detectable hairy cells and peripheral blood counts have increased to the normal range. This is different than definitions used by centers other than our own which define complete remission as being present when a single, or at most, two sequential unilateral iliac crest bone marrow biopsies show no evidence of involvement with hairy cell leukemia. Thus, our apparently lower than expected complete remission rate may be a reflection of the stringency with which we define this response. As mentioned above, an outside pathology consultant will be reviewing marrows from our patients as well as patients receiving single agent deoxycoformycin to determine if our response is indeed different than observed in other centers.

A significant problem that we have observed in patients receiving the combination of deoxycoformycin and interferon has been the development of depression. So far 5 patients have developed significant depression and two have required therapy with tricyclic antidepressants. Although depression can occasionally be seen in patients receiving alpha interferon (1 of 56 patients on our alpha interferon study in hairy cell leukemia has developed significant depression), the frequency with which we have observed it in patients receiving combination therapy is unexpectedly high.

SIGNIFICANCE

This study was undertaken primarily to improve the long-term response duration and the initial response rate of hairy cell leukemia patients. Deoxycoformycin is effective in patients with interferon-resistent hairy cell leukemia and it makes sense, therefore, to combine these two agents in an attempt to improve the overall response rate and duration. It appears, however, that from the preliminary data gathered so far that there will not be an improvement in either of these parameters although, clearly, longer follow-up will be necessary.

In addition, it appears that an unexpected toxicity, depression, might limit the applicability of this therapy to hairy cell leukemia patients in general even if long-term response duration is improved by the combination approach.

PROPOSED COURSE

We will be closing this trial shortly for the reasons mentioned above.

PUBLICATIONS

Foon, K.A., Maluish, A.E., Abrams, P.G., Wrightington, S., Stevenson, H.C., Alarif, A., Fer, M.D., Overton, W.R., Poole, M., Schnipper, E.F., Jaffe, E.S., Herberman, R.B. Recombinant leukocyte a interferon therapy for advanced hairy cell leukemia: Therapeutic and immunologic results. Am. J. Med. 80: 351-356, 1986.

Foon, K.A., Nakano, G.M., Koller, C.A., Longo, D.L., Steis, R.G. Response to 2'-deoxycoformycin after failure of interferon alpha in nonsplenecotomized patients with hairy cell leukemia. Blood, 68:297-300, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09292-02 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Adoptive Immunotherapy Utilizing LAK Cells & IL-2 Administered Intraperitoneally

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

PI:	R. G. Steis	Chief	CRB, NCI
	R. Ozols	Head, Exp. Therap. Sect.	COP, NCI
Others:	J. Ortaldo	Chief	LEI, NCI
	D. L. Longo	Associate Director	OAD, NCI
	M. T. Lotze	Investigator	COP, NCI

(See next page for continuation)

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (Walter Urba and Suzanne Beckner); Frederick Memorial Hospital (Carolyn Schoenberger).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☒ (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 study using autologous lymphokine activated killer cells administered intraperitoneally along with interleukin-2 has been initiated. We reasoned that for patients with cancer limited to the peritoneal cavity administering LAK cells and IL-2 into this anatomic compartment might bring about antitumor effects but with less toxicity than observed when this therapy is given systemically. Patients are staged and if found to have disease limited to the peritoneal cavity undergo priming with interleukin-2, pheresis, and infusions intraperitoneally of LAK cells and interleukin-2. Toxicity is similar to that observed in patients receiving systemic LAK and IL-2 and consists of fluid retention, hypotension, oliguria, rash, pruritis, malaise and fatigue, diarrhea, nausea and vomiting, and occasional episodes of thrombocytopenia. However, the degree of toxicity has been substantially less than patients we have treated at the BRMP with systemic LAK and IL-2. Two of 6 ovarian carcinoma patients have had partial responses, and 3 of 8 patients with colorectal carcinoma have had partial responses. Only microscopic disease was found at second-look laparoscopy or laparotomy in 3 of the 5 patients with partial responses. These 3 patients all had gross macroscopic disease prior to initiation of therapy, and 2 had lesions greater than 2 cm in diameter prior to therapy.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Robert Ozols	Head, Exp. Therap. Sect.	COP	NCI
John Ortaldo	Chief	LEI	NCI
Dan L. Longo	Associate Director	OAD	NCI
Michael T. Lotze	Investigator	COP	NCI
Stephen A. Rosenberg	Chief	SB	NCI
Michael Bookman	Senior Investigator	MB	NCI
Elizabeth Reed	Senior Staff Fellow	CIB	NCI
Jorge Carrassquillo	Head, Antibody Study Group	CCNM	NCI
Andrew Keenan	Head, Clinical Study Section	CCNM	NCI
Steven Larson	Chief	CCNM	NCI
Ami Ostega	Research Nurse	MB	NCI
Robert Young	Chief	MB	NCI

OBJECTIVES

1. To determine the toxicity of intraperitoneally administered LAK cells alone and of LAK cells plus interleukin-2.

2. To determine the antitumor activity of intraperitoneally administered LAK cells plus IL-2 in patients with epithelial ovarian cancer limited to the peritoneal cavity and peritoneal carcinomatosis arising from colorectal cancer.

3. To determine the in vivo trafficking of ^{111}In labeled IL-2 activated peripheral blood mononuclear cells by external scanning and by serial measurement of LAK cell activity in peripheral blood mononuclear cells after intraperitoneal administration of LAK cells.

4. To determine the pharmacokinetics of interleukin-2 in serum and peritoneal fluid after i.p. administration.

5. To determine the biologic effects on peripheral blood mononuclear cells of intraperitoneally administered IL-2.

METHODS EMPLOYED

Any patient with disease outside of the peritoneal cavity, for example lung, liver, or retroperitoneal nodal metastasis is excluded from study. Patients subsequently undergo peritoneoscopy at either the Clinical Center at the NIH or Frederick Memorial Hospital and a Tenckhoff catheter is placed. During placement of the Tenckhoff catheter, the abdomen is staged for the amount of disease and for the presence of malignant cells in washings or ascites.

Patients are primed with 100,000 units per kilogram of recombinant interleukin-2 intravenously every 8 hours for 3 consecutive days. Following two days of rest, the patients undergo a daily leukapheresis for 5 consecutive days.

The cells thus obtained are transferred to the LAK laboratory where they are purified by centrifugation over a cushion of Ficoll-Hypaque, and they are then incubated in interleukin-2 to generate LAK cells. After 7 days of incubation with the interleukin-2, the cells are washed and resuspended in interleukin-2 containing saline solution. The cells are transported to the bedside and are administered intraperitoneally. Interleukin-2 is administered along with the LAK cells intraperitoneally at a dose of 25,000 units per kilogram every 8 hours. Patients receive intraperitoneal infusions of LAK cells and interleukin-2 for five consecutive days (5 total infusions of LAK cells and 15 total doses of interleukin 2) and the following 2 weeks undergo pheresis and infusions of LAK cells and interleukin-2 as outlined previously. Patients are evaluated for response, thus, after four and a half weeks of therapy total. Responding patients have returned for repeat administrations of LAK cells and IL-2.

MAJOR FINDINGS

At the initiation of this study, 3 patients received intraperitoneal infusions of LAK cells daily for 5 consecutive days without the concurrent administration of interleukin-2. This was done to determine the toxicity of LAK cells alone since LAK cells had not been administered intraperitoneally to humans. One of these 3 patients developed significant diffuse abdominal pain requiring narcotic analgesia. This pain recurred after each dose of LAK cells but with less severity with each subsequent administration of cells. These 3 patients and all subsequent patients then received LAK cells and interleukin 2 intraperitoneally as outlined above. As mentioned, the toxicity observed is similar to that observed in patients receiving systemic LAK and IL-2 although the degree of toxicity is substantially less. These toxicities have included hypotension, in some instances requiring pressors for blood pressure support, oliguria, rash, pruritus, greater than 10 percent weight gain over initial body weight, fever, anorexia, unifocal PVCs, and one instance of atrial fibrillation, one instance of multifocal atrial tachycardia, chills, diarrhea, mild dyspnea related to elevated diaphragms, fatigue, headache, nausea and vomiting, thrombocytopenia, mild hyperbilirubinemia, mild increases in creatine levels, and eosinophilia. Toxicities unique to this method of administering LAK cells and IL-2 have included excessive abdominal distention resulting in some abdominal pain, rebound abdominal tenderness and a chemical profile of the peritoneal fluid suggesting a chemical peritonitis and dyspnea related to elevated diaphragms.

Clinical results so far in this trial have included partial responses in 2 of 6 evaluable patients with ovarian carcinoma and partial responses in 3 of 8 patients with colorectal carcinoma. In 3 of the partial responders, the response resulted in their being only microscopic residual disease found at the time of their second-look procedure. The second-look evaluation in all patients included either laparoscopy or laparotomy. The partial responders have come back for repeat administration of LAK cells and interleukin-2, but unfortunately in all 4 cases, there has been the development of significant fibrosis in the abdominal cavity which, although not associated with any clinical abnormalities, made it impossible for us to administer further therapy without an initial surgical resection of the adhesions.

Laboratory monitoring of this trial has indicated that sustained very high levels of interleukin-2 can be maintained in the abdominal cavity with this dose of interleukin-2 administered intraperitoneally (25,000 units per kilogram intraperitoneally every 8 hours). Levels have ranged from peak levels in the range of 100,000 units per milliliter to trough levels in the range of 5,000 to 8,000 units per milliliter. The concentration of interleukin-2 required in vitro to maintain LAK cell activation is approximately 1,000 units per milliliter, and clearly it would be possible in the future to reduce the dose of interleukin-2 to provide adequate levels to maintain LAK cell activation. The concentration gradient from the intraperitoneal space of the serum of interleukin-2 has been on the order of 100 to 1. We have also been able to show that gamma interferon is produced by cells in the peritoneal cavity resulting in levels in ascites fluid of up to 200 units per milliliter. Monocytes isolated from the peritoneal cavity have been shown to be activated, at least as measured by their increased production of hydrogen peroxide. Whether these activated monocytes participate in the antitumor effects observed in patients receiving this therapy so far is not clear. In addition, it has been possible to show that as long as interleukin-2 is administered intraperitoneally, LAK activity is recoverable from the peritoneal cavity. When we compared LAK recovery in patients receiving interleukin-2 intraperitoneally to those receiving no interleukin-2 intraperitoneally (i.e. the first 3 patients on the trial as mentioned above), it was obvious that interleukin-2 was playing a critical role in maintaining intraperitoneal LAK activity. When interleukin-2 is not administered, LAK activity is not recoverable after administration of the LAK cells.

SIGNIFICANCE

This is the first study to show that intraperitoneal therapy with LAK cells and interleukin-2 is capable of bringing about significant antitumor effects in the peritoneal space and with acceptable toxicity. The major limiting toxicity to further administration of this therapy is not patient tolerance but the development of intraperitoneal fibrosis. This intraperitoneal fibrosis results in compartmentalization of the abdomen such that it is impossible to expose the entire peritoneal cavity to immunotherapy. The immunologic monitoring performed on these patients has shown us that it would be possible to administer lower and better tolerated doses of interleukin-2 to these patients intraperitoneally and yet still be able to maintain intraperitoneal LAK activity.

PROPOSED COURSE

There are several options that one could use at this point to try to limit the toxicity including the intraperitoneal fibrosis in future patients. Since interleukin-2 itself might be involved with the pathogenesis of the fibrosis, it might be possible to reduce the dose of interleukin-2 by providing an initial loading followed by a continuous infusion. The pharmacokinetic data obtained so far is being analyzed by personnel of the Pharmacology Branch, and we will soon be starting a second-generation trial which will use continuous infusion intraperitoneal interleukin-2 rather than bolus interleukin-2 to maintain intraperitoneal LAK activity. Gamma interferon might also be used to prevent intraperitoneal fibrosis and, hopefully, also augment the therapeutic

activity of this regimen. Data obtained by Dr. Rosenberg and by ourselves in patients receiving systemic LAK cells and IL-2 indicates that the induction of HLA-Dr expression on tumor cells correlates with antitumor response to systemic LAK and IL-2. Ovarian cancer cells are known to express HLA-Dr and in all probability the expression of HLA-Dr can be augmented by administration of gamma interferon. In the new intraperitoneal LAK and IL-2 trial in addition to providing continuous infusion interleukin-2 we will pretreat the abdominal cavity with intraperitoneal gamma interferon. We thereby hope to up regulate HLA-Dr expression on the tumor cells in an attempt to bring about a greater antitumor effect with the subsequently administered LAK cells and interleukin-2. Gamma interferon also has the interesting and fortunate effect of down-regulating secretion of procollagen type 1 and 3 from fibroblast. Since procollagen types 1 makes up to 80 percent of all collagen in the body, if we could significantly decrease type 1 and type 3 collagen synthesis in the peritoneal cavity by preadministering gamma interferon, we might be able to prevent the development of intraperitoneal fibrosis. Colchicine has been used in animal models to prevent the development of experimental intraperitoneal fibrosis. We have added colchicine to cultures of LAK cells and have found that concentrations up to 10^{-8} molar do not inhibit the generation of LAK cells or LAK activity. Colchicine will be administered intraperitoneally to patients receiving this therapy in a further attempt to reduce the development of intraperitoneal fibrosis.

Thus, we intend to press on with intraperitoneal LAK and IL-2 therapy but try to modify the toxicities observed in the initial trial with the modifications outlined above.

PUBLICATIONS

Steis, R., Bookman, M., Clark, J., Urba, W., McKnight, J., Smith, J., Schoenberger, C., Ozols, R., Young, R., Maluish, A., Beckner, S., and Longo, D.: Intraperitoneal lymphokine activated killer (LAK) cell and interleukin-2 (IL-2) therapy for peritoneal carcinomatosis: Toxicity, efficacy, and laboratory results. Proc. ASCO Annual Meeting, May 17-19, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09294-02 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Treatment of Relapsed T Cell Lymphomas with Recombinant Leukocyte A Interferon

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

PI:	R. G. Steis	Chief	CRB, NCI
CoPI:	D. Ihde	Senior Investigator	NMOB, NCI
Others:	J. D. Minna	Chief	NMOB, NCI
	E. Sausville	Senior Investigator	NMOB, NCI
	J. Clark	Senior Staff Fellow	CRB, NCI
	J. Smith	Senior Staff Fellow	CRB, NCI
	R. Miller	Senior Staff Fellow	CRB, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (Walter Urba, Edward Crum).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Previous trials have demonstrated activity of recombinant alpha interferon in patients with cutaneous T cell lymphomas. The initial trials used interferon at its maximal tolerated dose, that is, 50 million units per meter squared given three times per week. Most patients on this study required dose reduction because of excessive toxicity; and although partial responses were seen in approximately 50 percent of patients, they were of limited duration, and no complete responses were seen. This trial is an attempt to increase the complete response rate and the response duration by administering high doses of interferon daily for five consecutive days followed by a two-week rest period. Also in this study, it was hoped to increase the dose in those patients who were not responding or who had only a partial response.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Dan Ihde	Senior Investigator	NMOB	NCI
John D. Minna	Chief	NMOB	NCI
Edward Sausville	Senior Investigator	NMOB	NCI
Jeffrey Clark	Senior Staff Fellow	CRB	NCI
John W. Smith	Senior Staff Fellow	CRB	NCI
Robin Miller	Senior Staff Fellow	CRB	NCI

OBJECTIVES

1. To study the clinical efficacy of a high-dose intermittent schedule of recombinant alpha interferon in patients with T cell lymphoma.

2. To determine the safety of this schedule of interferon adminstration.

METHODS EMPLOYED

Previous studies using recombinant alpha interferon in patients with relapsed cutaneous T cell lymphoma have shown significant activity of this drug. However, the dose used was the maximal tolerated dose, 50 million units per m^2 administered intramuscularly three times per week continuously. Dose reductions were necessary in all patients entering this trial because of excessive toxicity. Although partial responses occurred in approximately 50 percent of patients, they were of fairly short duration, and no complete responses were observed.

The results of this study suggested that although interferon administered via this dose and schedule was active, dose limiting toxicity prevented the continuous administration of full doses. Perhaps higher response rates and better tolerence of the therapy could be obtained if the schedule of adminstration was altered. The dose and schedule to be used in this study, therefore, was changed so that patients would receive 10 million units per m^2 intramuscularly on day 1 followed by 50 million units per m^2 on days 2 through 5. This schedule of administration would then be repeated every three weeks. It is hoped that by altering the schedule of interferon administration as outlined in this protocol that more drug might be administered and dose reductions would not be required as often. The escalation of the dose to 100 million units/m^2 on days 2 through 5 in patients with at least stable disease on the lower dose after 3 cycles would test the hypothesis that there is a dose response relationship in patients with cutaneous T-cell lymphoma to interferon.

MAJOR FINDINGS

The BRMP has admitted 8 patients to this study so far, 6 of these being evaluable for response. Of these, there have been 2 partial responses, and 4 patients have not responded to therapy. The toxicities experienced by patients on this study have been those expected from recombinant alpha interferon and include fatigue, anorexia, fever, chills, myalgias, headache,

and granulocytopenia. There were several episodes of nausea and vomiting, and one patient had an episode of confusion that resolved spontaneously.

This study is being conducted in conjunction with the Navy Medical Oncology Branch of the National Cancer Institute, and although the number of patients admitted in our trial does not allow us an adequate evaluation of the results of the total study, it appears from the results obtained in all patients admitted to this study so far that this method of administration of recombinant alpha interferon may be less active than the 3 times per week schedule administered. Toxicity may be slightly reduced, however.

SIGNIFICANCE

The significance of this study is that higher doses of alpha interferon in patients not responding to lower doses in this particular disease does not result in a higher rate of conversion of stable patients to responding patients. In addition, it appears that a q3 weekly administration of high doses of alpha interferon does not result in a higher incidence of response than 3 times per week therapy.

PROPOSED COURSE

Because this particular method of administration of alpha interferon has not resulted in a significant increase in the therapeutic effect in this disease, the study will shortly be closed.

PUBLICATIONS

Ihde, D.C., Steis, R., Sausville, E.A., Veach, S.R., Eddy, J., Bunn, P.A.: Phase II trial of intermittent high dose recombinant interferon alfa-2A (IFN-ALFA) in Micosis Fungoides and Sezary Syndrome (MF/SS). Abst. Proc. Am. Assoc. for Cancer Research, Vol. 28, p. 208, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09295-02 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Recombinant Leukocyte A Interferon in Non-Hodgkin's Lymphoma Patients

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

PI:	R. G. Steis	Chief	CRB, NCI
Others:	R. Simon	Chief, Biometric Research Br.	CTEP, NCI
	J. W. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith	Senior Staff Fellow	CRB, NCI
	M. Hawkins	Head, Biological Eval. Sect.	CTEP, NCI
	D. L. Longo	Associate Director	OAD, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (W. Urba & E. Crum); U. of Michigan, Ann Arbor, MI (K. Foon); Johns Hopkins U., Baltimore, MD (S. Staal); GW U., Washington, DC (R. Siegel); Frederick Memorial Hospital (T. Watson).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Because low doses of recombinant alpha interferon are very effective in the treatment of patients with hairy cell leukemia, this study was designed to determine if equally low doses of alpha interferon were effective in another indolent B-cell neoplasm, favorable histology non-Hodgkin's lymphomas. We also wish to determine the effects of recombinant alpha interferon in low doses in patients with the T-gamma lymphoproliferative disorder, a disease of T-cells that resembles in many regards hairy cell leukemia.

Thirty-six patients with favorable histology chemotherapy-resistant non-Hodgkin's lymphoma were randomized, sixteen to the low-dose arm of the study (3 million/units qd) and twenty to the high-dose arm of the study (50 million units I.M. biw). The overall response rate is 26 percent with equal percentages of responders in each arm of the study. Of six patients with the T-gamma lymphoproliferative disorder, none have had an obvious antitumor response. However, one patient with pure red cell aplasia no longer has absence of red cell precursors in this marrow and no longer is requiring transfusion support.

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

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Richard Simon	Chief, Biometric Research Br.	CTEP	NCI
Jeffrey W. Clark	Senior Staff Fellow	CRB	NCI
John W. Smith	Senior Staff Fellow	CRB	NCI
Robin L. Miller	Senior Staff Fellow	CRB	NCI
Michael Hawkins	Head, Biological Eval. Sect.	CTEP	NCI
Dan L. Longo	Associate Director	OAD	NCI

OBJECTIVES

1. To study the clinical efficacy of recombinant alpha interferon administered in two different schedules and doses in patients with favorable histology non-Hodgkin's lymphoma.

2. To determine the tolerance of patients to these two different schedules and doses of interferon alpha administration.

3. To study the therapeutic efficacy of recombinant alpha interferon at high doses in patients with diffuse poorly differentiated lymphoma.

4. To study the therapeutic efficacy of recombinant interferon alpha in patients with chronic T gamma lymphoproliferative disorder.

METHODS EMPLOYED

Previous studies at the BRMP and elsewhere have demonstrated significant activity of recombinant alpha interferon in high doses in patients with favorable histology non-Hodgkin's lymphoma and possibly in patients with diffuse poorly differentiated lymphoma. However, patients treated on these studies received interferon at maximally tolerated doses, and all patients required dose reduction. The responses that were observed were of short duration, and most responses were partial responses. Studies done elsewhere indicated that very low doses of recombinant alpha interferon were very effective in bringing about responses in another low-grade lymphoproliferative disorder, hairy cell leukemia. We, therefore, wished to determine if lower and possibly better tolerated doses of recombinant alpha interferon would be as effective as the higher and more toxic doses used in the initial studies. In addition, the initial study demonstrated responses in patients with diffuse poorly differentiated lymphoma. Only small numbers of these patients had been treated and a second objective of the study was therefore to treat further patients with this histology with the original high doses of interferon used in the initial study to further define their responses.

A recently described lymphoproliferative disorder termed the T-gamma lympholiferative disorder is a disease of T cells that clinically resembles hairy cell leukemia. These patients typically present with pancytopenia and splenomegaly and a history of tranfusion requirements and infections. This

disease is due to proliferation of a cell that bears receptors for the Fc portion of IgG, and frequently this cell also has a large granular lymphocyte morphology, and bears the T-cell associated antigens OKT 3 and OKT 8. Because this disease has no consistently effective and safe treatment, and because it resembles in many respects hairy cell leukemia, we wish in this study to determine the efficacy of low doses of recombinant alpha interferon in this disease.

For patients with low-grade non-Hodgkin's lymphoma an evaluation for the extent of disease is completed prior to the administration of therapy. Patients are then randomized to receive either 3 million units of recombinant alpha interferon subcutaneously daily or 50 million units per m^2 of recombinant alpha interferon intramuscularly twice per week. Patients receive therapy for three consecutive months and at that time are evaluated for response and toxicity. If there is no response, patients are taken off study. If a response has occurred, they will continue to receive interferon at the initial dose as long as the response continues or until toxicity necessitates drug withdrawal.

Patients with diffuse poorly differentiated lymphoma are evaluated for disease extent and then are started in a nonrandom fashion on recombinant alpha interferon at a dose of 50 million units per m^2 given intramuscularly three times per week. Patients continue on therapy for three consecutive months, and responders will continue on therapy at the highest tolerated dose. Nonresponders are removed from study at three months.

Patients with the T gamma lymphoproliferative disorder are evaluated prior to the initiation of therapy. If they are free from infection, interferon is administered at a dose of 3 million units daily subcutaneously. Patients are taught to self-administer the drug and are discharged to home. They are evaluated in the clinic at monthly intervals for any evidence of response.

MAJOR FINDINGS

So far, 36 patients with favorable histology non-Hodgkin's lymphoma have been randomized on the study, 16 to the low-dose arm of the study and 20 to the high-dose arm of the study. There was 1 complete response and 2 partial responses among the 16 patients randomized to the low-dose arm of the study, and 2 partial responses and 1 complete response among those patients randomized to the high-dose arm of the study. Thus, the overall response rate of 26 percent was equally represented in both arms of the study. The response duration was shorter in the low-dose arm. Thus, the preliminary data would suggest that there is no advantage to administering high-dose therapy to these groups of patients. Toxicity observed so far has been qualitatively the same in both arms of the study; however, it has been much more severe in the high-dose group.

Among patients with the T-gamma lymphoproliferative disorder, none of 6 patients have responded to therapy. One patient as described above has had resolution of transfusion requirements that is ongoing at 10 months although he has had no demonstrable antitumor effects so far.

SIGNIFICANCE

The results of this study so far suggest that there is no definite dose

response relationship in patients with non-Hodgkin's lymphoma being treated with alpha interferon at least between the two doses studied. Clearly, the lower dose of interferon is much better tolerated than the high dose of interferon and might be preferable for use in patients with heart disease or with other reasons for which high-dose interferon therapy cannot be given. It does not appear, however, that recombinant alpha interferon will have a major role in the future therapy of this group of neoplasms because of the fairly low overall response rate and short response duration.

Despite the close clinical resemblance of the T-gamma lymphoproliferative disorder to hairy cell leukemia, it does not appear that a high incidence response to alpha interferon can be expected in this disease. The observation that 1 patient with pure red cell aplasia can have reversal of his need for red cell transfusion support without concurrent resolution of malignant cells in his marrow or peripheral blood suggest that the pure red cell aplasia is mediated by a product made directly or indirectly by the T-gamma cells. Although his response to therapy has been of major benefit to the patient, the most interesting study of this patient to understand the mechanism of action of interferon in this disease has yet to be performed. In vitro studies of this patient's marrow specimens before and after interferon therapy might help shed light on the mechanism of action of interferon in this disease. These studies are now ongoing in the laboratory of Frank Ruscetti.

One aspect of immunologic therapy that has not been adequately explored so far has been the use of standard forms of chemotherapy with biological response modifiers. Clearly, combination chemotherapy is very effective in patients with low-grade non-Hodgkin's lymphomas. Because alpha interferon can bring about regressions of this tumor in patients with chemotherapy-resistent disease, it might be that combinations of alpha interferon and chemotherapy might be of greater efficacy than chemotherapy alone in the treatment of these diseases. Preliminary trials using combinations of chemotherapy with alpha interferon have demonstrated significant myelosuppressive effects of the combination that seem greater than what would be expected in response to either interferon or chemotherapy alone. If one wishes to combine chemotherapy with interferon, it appears that lower doses of interferon would be better than higher doses of interferon since patients would be unlikely to tolerate higher doses of interferon with chemotherapy because of excessive myelosuppression. The data generated on this trial so far would suggest that one could rationally combine low doses of interferon with standard-dose chemotherapy in the treatment of this disease.

PROPOSED COURSE

The results of the trials so far are being analyzed in detail. If a statistical analysis of this data reveals that accrual of additional patients will add no more useful information to this study, the study will be closed.

PUBLICATIONS

Steis, R. G., Foon, K. A., and Longo, D. L.: Current and future uses of recombinant interferon alpha in the treatment of low grade non-Hodgkin's lymphoma. Cancer 59(3): 658-663, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09298-02 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Adoptive Immunotherapy of Cancer with Autologous LAK Cells Plus IL-2

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

PI:	S. Rosenberg	Chief	SB, NCI
Frederick Supervisor -	D. L. Longo	Associate Director	OAD, NCI
Others:	L. Muul	Cancer Expert	MB, NCI
	M. Lotze	Investigator	COP, NCI
	R. G. Steis	Chief	CRB, NCI

See next page for continuation

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (Walter Urba and Edward Crum).

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (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.)*

Exposure of peripheral blood mononuclear cells from cancer patients to high concentrations of recombinant IL-2 in vitro has been observed to result in the generation of cells capable of causing lysis of a variety of tumor cells but not of normal lymphocytes. This study is an attempt to utilize such cells in the therapy of cancer in humans. Previous studies in the Surgery Branch of the National Cancer Institute demonstrated significant antitumor activity of these cells when administered concurrently with IL-2. However, toxicity was substantial, and all patients required the administration of this therapy in the intensive care unit setting. Thus, in the first 11 patients entered on this study at Frederick, intermediate doses of dexamethasone were administered concurrent with the therapy in an attempt to reduce associated toxicity. The initial result suggested that there was a reduction in the antitumor activity when steroids were administered concomitantly. As a result, subsequent patients have been treated without steroids.

To date, among 27 patients evaluable for response on this study, there have been 2 patients with a partial response. One was in a patient with a malignant melanoma and the other in a patient with nodular mixed lymphoma that had undergone a histologic conversion to a diffuse large cell lymphoma. Toxicity has been substantial.

PROJECT DESCRIPTION

PERSONNEL

Steven Rosenberg	Chief	SB	NCI
Dan L. Longo	Associate Director	OAD	NCI
Linda Muul	Cancer Expert	MB	NCI
Michael Lotze	Investigator	COP	NCI
Ronald Steis	Chief	CRB	NCI
JoAnn Beman	Nurse Specialist	CRB	NCI
Colleen Simpson	Nurse Specialist	MB	NCI
Jeffrey Clark	Senior Staff Fellow	CRB	NCI
Robin Miller	Senior Staff Fellow	CRB	NCI
John Smith	Senior Staff Fellow	CRB	NCI
Stephen Creekmore	Chief	BRB	NCI

OBJECTIVES

To confirm and extend the observations of Dr. Steven Rosenberg and his colleagues on the efficacy of systemically administered autologous lymphokine activated killer cells plus interleukin-2.

METHODS EMPLOYED

Patients considered eligible for this study are evaluated in the outpatient clinic, and an assessment is made of their ability to tolerate the therapy. If admitted to the study, patients are given 100,000 units per kilogram of interleukin-2 every 8 hours for a mximum of 9 doses. They receive no subsequent IL-2 for two further days and then undergo a leukapheresis once daily for 5 consecutive days. The cells obtained after each leukapheresis are sent to the laboratory where they are purified and cultured in the presence of recombinant interleukin 2. After the fifth leukapheresis on a Friday, patients are admitted to the intensive care unit where extensive monitoring can be performed during the course of therapy. Interleukin-2 is administered at a dose of 100,000 units per kilogram every 8 hours and is given as long as the patient can tolerate it. Cells obtained from Monday and Tuesday are infused on Friday. Cells obtained from Wednesday are infused on Saturday, and cells obtained on Thursday and Friday are infused on the following Monday. Again, an attempt is made to administer this dose of recombinant interleukin-2 for as long as patients can tolerate the therapy. In general, patients are able to tolerate therapy until Monday or Tuesday, and then interleukin-2 must be stopped. Patients are allowed to recover from the side effects, and the cycle of leukapheresis followed by infusion of cells and interleukin-2 is repeated starting the following Monday.

In an attempt to reduce the toxicity of this therapy, moderate doses of dexamethasone, 2 milligrams every 6 hours, were administered intravenously concurrent with the start of LAK cell and IL-2 administration. After 9 consecutive patients had been treated in this manner (the lymphoma patients treated on the study did not receive steroids), it was felt that perhaps responses were not being seen as freqently as in the previous Surgery Branch Study. Therefore for all subsequent patients, steroids were not administered.

MAJOR FINDING

Twenty-seven patients have now been admitted to this study and there have been 2 partial responses observed. One partial response lasted for 4 months, the other for 10 months. The study has been amended so that only patients with non-Hodgkin's lymphomas and Hodgkin's disease refractory to standard therapy are now admitted. Among 6 patients with these 2 diagnoses, there has only been 1 partial response.

As expected, toxicity has been substantial and includes greater than 10 percent weight gain, hypotension requiring pressors, oliguria, rash with pruritis, edema, fever, anorexia, cardiac arrhythmias, chills, confusion, depression, diarrhea, fatigue, skin desquamation, headache, stomatitis, myalgias, nausea and vomiting, thrombocytopenia, hyperbilirubinemia, elevations in serum creatinine, eosinophilia, and one patient had a grand mal seizure.

SIGNIFICANCE

This study demonstrates that the technology for generation of LAK cells and administration of LAK cells and IL-2 can be performed outside of the Surgery Branch. Our response rate is somewhat lower than that in the initially reported study but we have not been excluding patients based upon bulk of disease, and lower levels of performance status have been accepted in our patients going on study.

PROPOSED COURSE

The institution of a series of extramural studies in renal cell cancer, melanoma, and colon cancer makes our effort in these areas somewhat less important. We intend to continue to focus our patient accrual on patients with Hodgkin's disease and the non-Hodgkin's lymphomas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09304-01 CRB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Eval. of Human Anticolorectal Carcinoma MoAb in Pats. with Dissem. Colon CA

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

PI:	R. G. Steis	Chief	CRB, NCI
CoPI:	M. Bookman	Senior Staff Fellow	MB, NCI
Others:	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. L. Miller	Senior Staff Fellow	CRB, NCI
	J. Clark	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI

See next page for continuation

COOPERATING UNITS *(if any)*
PRI, NCI-FCRF (W. Urba and E. Crum); Bionetics Research, Inc., Gaithersburg, MD (R. McCabe, M. Hanna, and M. Hospel); Frederick Memorial Hospital (Valarie Dailey).

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

A large number of patients have been treated with murine monoclonal antibodies in several phase I and II studies. In general, the anti-tumor effects of this therapy have been disappointingly few. It has been demonstrated, however, that antibodies can reach the site of the tumor and stain the tumor cells specifically. In general it appears that monoclonal antibodies in and of themselves as currently being used are not effective tools in the treatment of cancer.

One limiting feature of the use of murine monoclonal antibodies has been the development of human antimouse antibodies. If antibodies ultimately are modified to become an effective antitumor treatment for patients with cancer, the development of human antimouse monoclonal antibodies will still be a significant problem. We, therefore, initiated a study using human monoclonal antibodies in an attempt to treat patients with colorectal carcinoma. Two human monoclonal antibodies 16.88 and 28A32 are currently being studied. Both are derived from human B cells from patients with colorectal carcinoma immunized with an autologous tumor cell vaccine. These monoclonal antibodies have been radiolabeled with I-131 and have localized to tumors in patients with metastatic colorectal carcinoma. Of 14 patients treated so far, none have developed antibodies against the administered human monoclonal antibody. However, two patients had pre-existing antibody directed against either or both monoclonal antibodies. These patients were not entered on the study. Despite trafficking of these monoclonal antibodies to the tumor-bearing site, no antitumor responses have been observed.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Michael Bookman	Senior Staff Fellow	MB	NCI
John W. Smith II	Senior Staff Fellow	CRB	NCI
Robin L. Miller	Senior Staff Fellow	CRB	NCI
Jeffrey Clark	Senior Staff Fellow	CRB	NCI
Stephen Creekmore	Chief	BRB	NCI
Carl Pinsky	Chief Medical Officer for Extramural Research	OAD	NCI
Dan L. Longo	Associate Director	OAD	NCI
Jorge Carrassquillo	Head, Antibody Study Group	CCNM	NCI
Andrew Keenan	Head, Clinical Study Section	CCNM	NCI
Steven Larson	Chief	CCNM	NCI
James Reynolds	Medical Officer	CCNM	NCI

OBJECTIVES

1. Determine the in vivo toxicity, pharmacokinetics, tissue localization, and potential therapeutic benefit of 16.88 and 28A32 monoclonal antibodies in patients with colorectal carcinoma.

2. To attempt radioimaging with the antibody conjugated to ^{131}I, to study the biodistribution of the antibody, to study the effects of an increase of cold antibody on its biodistribution, and finally to determine whether the antibody will have diagnostic utility when conjugated to an isotope.

METHODS EMPLOYED

Prior to referral, tumor tissue from patients previously undergoing resection of colorectal carcinoma is tested for reactivity with both monoclonal antibodies. If the tumors are reactive with one or both antibodies, patients are evaluated in the clinic for entrance into the study. If the performance status is greater than 70 percent and measurable disease is present, patients are injected on week 1 with 8 mg of the appropriate monoclonal antibody radiolabeled with 5 millicuries of ^{131}I. In week 2 the same amount of labeled antibody is administered together with "cold" antibody of increasing amounts in consecutive groups of 3 patients. In weeks 3 through 5, "cold" antibody is administered alone in an attempt to determine if the antibody can bring about an antitumor effect alone. Previous studies with other antibodies have shown that when excess cold antibody is administered to patients receiving labeled antibody, localization of the radiolabeled antibody to sites of tumor can be enhanced.

MAJOR FINDINGS

Six patients have now received antibody 16.88 and 8 patients antibody 28A32. No toxicity has been observed. No patient developed antibodies against the administered human monoclonal antibody. All but one patient with tumors larger than 4 cm had positive images of at least 1 tumor-bearing site. Tumor deposits less than 4 cm in diameter were not imaged. Contrast between tumor and

non-tumor sites increased until approximately day 6 and images of tumors persisted for up to 2 weeks after initial injection of the antibody. No antitumor responses were observed.

One patient who was injected with 28A32 had none of multiple tumor deposits imaged. This one patient was found to have an endogenous antibody that reacted with the same antigen recognized by 28A32. Two patients were not entered on the study because of baseline skin test reactivity to intradermally administered monoclonal antibody. The basis for this skin test response is not known.

SIGNIFICANCE

This study has pointed out potential advantages and potential problems associated with the administration of human monoclonal antibodies. First, the antibody is clearly not immunogenic as none of our patients treated with the antibody have developed antibody to the administered monoclonal. It would appear, therefore, that if an effective way of administering the antibody could be determined, repeated administration of the antibody would not be prevented by the development of a humoral immune response. There has not been any toxicity associated with the administration of this antibody. It appears, however, that occasional patients will have circulating endogenous antibody reactive with the same antigen recognized by the administered monoclonal. In addition, there may be patients who will have baseline reactivity to the antibody even though they never previously had been exposed to it.

PROPOSED COURSE

Subsequent groups of 3 patients will receive doses of monoclonal antibody up to a total dose of 200 milligrams. Anti-idiotypic monoclonal antibodies have recently been developed that recognize the idiotypes on both 16.88 and 28A32. We plan to perform tumor and non-tumor biopsies from the livers of patients receiving both antibodies and stain the tumors with anti-idiotypic antibody. Using this test we hope to verify specific localization of antibody to the tumor. In addition, we hope to administer an ^{125}I labeled irrelevant human monoclonal antibody along with either 16.88 or 28A32. Tumor biopsies will be obtained from patients receiving both antibodies and a determination made as to the relative amounts of ^{125}I and ^{131}I in tumor bearing site and non-tumor bearing sites. In this way, we hope to determine if localization of the antibody to the tumor site is specific as opposed to being a reflection of leakage of a radiolabeled antibody through the abnormal vasculature of tumor deposits.

PUBLICATIONS

Steis, R., Smith J., Bookman, M., Carrasquillo, J., Larson, S., Reynolds, J., Dailey, V., Perentesis, P., Urba, W., McKnight, J., Clark, J., McCabe, R., Hanna, M., Haspel, M., Longo, D.: Evaluation of a human anti-colorectal carcinoma monoclonal antibody in patients with metastatic colorectal cancer. Proc. Second Inter. Conf. on Monoclonal Antibody Conjugates, March 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09305-01 CRB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
2'dCF in IFN-Resistant Hairy Cell Leukemia or T Gamma Lymphoprolif. Disorder

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

PI:	R. G. Steis	Chief	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. L. Miller	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI

See next page for continuation

COOPERATING UNITS *(if any)*
PRI, NCI-FCRF (Walter Urba); Frederick Memorial Hospital (Nancy Englar).

LAB/BRANCH
Clinical Research Branch

SECTION

INSTITUTE AND LOCATION
NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The BRMP has an ongoing study of recombinant alpha interferon in patients with hairy cell leukemia. A number of these patients have developed disease progression despite continued administration of interferon. Administration of higher doses of interferon has resulted in only minor and transient further tumor responses. This trial was designed as a salvage trial for these patients.

Three patients have been admitted to this trial so far and 2 of these have achieved a partial response. All 3 patients had significant cytopenias when they started the study and all were refractory to interferon. The 2 partial responses occurred very rapidly after administration of deoxycoformycin but were associated with transient episodes of severe thrombocytopenia and febril neutropenia. One patient died after 2 doses of deoxycoformycin due to refractory and transfusion-resistant thrombocytopenia. These partial responses have been substantial with only minimal, scant hairy cells identifiable in bilateral iliac crest bone marrow biopsies.

One patient with the T-gamma lymphoproliferative disorder has so far been treated on this study. This patient has not responded to date.

Toxicity associated with administration of dCF has included significant myelosuppression, fatigue, nausea, and vomiting.

PHS 6040 (Rev. 1/84) GPO 914-916

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Dan L. Longo	Associate Director	OAD	NCI
Jeffrey Clark	Senior Staff Fellow	CRB	NCI
John W. Smith II	Senior Staff Fellow	CRB	NCI
Robin L. Miller	Senior Staff Fellow	CRB	NCI
Stephen Creekmore	Chief	BRB	NCI
Allison Martin	Staff Fellow	DEB	NCI
Stacy Nerenstone	Staff Fellow	DEB	NCI
Seth Steinberg	Acting Head	CDMS	NCI

OBJECTIVES

1. To determine the clinical effects of low doses of 2' deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease refractory to or intolerant of low-dose alpha interferon.

2. Determine the biochemical consequences of the administration of 2'deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease.

3. To determine the immunologic consequences of the administration of deoxycoformycin in patients with hairy cell leukemia or T-gamma lymphoproliferative disease.

4. To study the effects of this therapy on the serum level of a soluble form of the IL-2 receptor.

METHODS EMPLOYED

Patients with hairy cell leukemia on our ongoing trial using single-agent alpha interferon for this disease are eligible for this study if they have progressive disease during the course of interferon administration. Patients with hairy cell leukemia are also eligible for the study if the side effects to alpha interferon on this initial study are intolerable. Patients with T-gamma lymphoproliferative disorder are eligible for this study if they have not responded to 3 consecutive months of administration of alpha interferon.

Once patients are deemed eligible for the study, they receive 4 mg/m^2 of deoxycoformycin intravenously every week for 3 consecutive weeks. Patients then receive 4 mg/m^2 on alternating weeks. Patients will receive this therapy for a minimum of 12 weeks, and if disease has stabilized, continued therapy will be given for at least 6 months unless disease progression or prohibitive toxicity occurs. If a patient achieves a complete pathologically documented remission, therapy will be continued for 3 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 6 month time point.

Deoxycoformycin is administered after hydration with 1 liter of D5 and half normal saline. After administration of the deoxycoformycin, one further liter of fluid is given intravenously.

MAJOR FINDINGS

Three patients with hairy cell leukemia resistant to alpha interferon have been admitted to the study so far. One patient died of refractory and transfusion-resistant thrombocytopenia. Two patients have had significant partial responses with only minor and scant involvement of the marrow with hairy cells after therapy. One patient with the T gamma lymphoproliferative disorder refractory to interferon is in her second month of therapy with this drug. So far, no definite response has been observed.

Toxicity observed so far has included severe myelosuppression including thrombocytopenia and neutropenia with the subsequent development of bleeding and fever. Fatigue, nausea, and vomiting have also occurred.

SIGNIFICANCE

Two effective therapies for hairy cell leukemia currently exist, alpha interferon and deoxycoformycin. Only small numbers of patients with interferon-resistant hairy cell leukemia have received deoxycoformycin. The response observed so far to this drug in interferon-resistant patients has been gratifying. The published literature and our own results, however, suggest that this subgroup of patients with hairy cell leukemia might be somewhat resistant to deoxycoformycin.

PROPOSED COURSE

We will continue to accrue patients to this study from our intramural interferon study. No modifications in this study are anticipated.

PUBLICATIONS

Foon, K.A., Nakano, G.M., Koller, C.A., Longo, D.L., Steis, R.G. Response to 2'-deoxycoformycin after failure of interferon alpha in nonsplenecotomized patients with hairy cell leukemia. Blood, 68:297-300, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09306-01 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Phase I Evaluation of Recombinant Human Granulocyte Macrophage CSF

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

PI:	R. G. Steis	Chief	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. L. Miller	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (Walter Urba and Edward Crum); Frederick Memorial Hospital (Jean Hersey).

LAB/BRANCH

Clinical Research Branch

SECTION

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NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Colony stimulating factors are a group of cytokines that have the desirable property of enhancing marrow production of granulocytes and macrophages. These cytokines might be of great interest to oncologists if they are capable of increasing peripheral blood granulocyte counts without significant associated toxicity in patients receiving myelosuppressive therapy. This study is a phase I evaluation of recombinant human GM-CSF. Patients with solid tumors with reasonably intact bone marrows will receive escalating doses of GM-CSF in an attempt to determine the maximally tolerated dose and the dose at which significant increases in peripheral blood granulocyte counts and granulocyte and monocyte function can be observed.

Only 1 patient has been admitted to this trial to date, and no comment can yet be made as to toxicity or antitumor effectiveness.

GPO 914-916

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB NCI
Dan L. Longo	Associate Director	OAD NCI
Jeffrey Clark	Senior Staff Fellow	CRB NCI
John W. Smith II	Senior Staff Fellow	CRB NCI
Robin L. Miller	Senior Staff Fellow	CRB NCI
Stephen Creekmore	Chief	BRB NCI

OBJECTIVES

1. To determine the toxic effects of recombinant human GM-CSF in patients with cancer.

2. To determine the effects of recombinant human GM-CSF on granulocyte and monocyte function in vivo.

3. To determine the in vivo effects of recombinant human GM-CSF on marrow cellularity, peripheral white blood cell counts and circulating and marrow CFU-GM and CFU-GEMM.

4. To determine the pharmacokinetics of IV administered recombinant human GM-CSF.

5. To determine the antitumor effects of recombinant GM-CSF.

METHODS EMPLOYED

Patients with solid tumors refractory to standard therapy are evaluated and staged as outpatients in the BRMP clinic. Once appropriate tumor measurements and staging has been performed, patients receive a single injection of recombinant human GM-CSF. Multiple blood samples are drawn to determine the pharmacokinetics of this drug, and patients are observed for 4 days for the development of toxic and hematologic effects. At that time, if no toxic effects have developed, patients receive daily injections of the same dose of recombinant human GM-CSF for 21 consecutive days. During this time, patients are evaluated for the development of toxicity and for a number of immunologic parameters including up-regulation of the expression of Mo-1 and Leu-M5 on granulocytes, changes in HLA-Dr expression on monocytes, changes in the rate of phagocytosis by granulocytes of fluorescein-labeled latex beads, changes in hydrogen peroxide generation by peripheral blood monocytes, changes in the cytotoxicity of peripheral blood monocytes against a melanoma cell line, A375, and changes in CFU-GM and CFU-GEMM in peripheral blood and bone marrow cells. At the completion of the 21 days of administration of GM-CSF, patients are evaluated for a tumor response and if stable are evaluated again in 1 month's time.

MAJOR FINDINGS

Only one patient has been admitted to the study so far, and no toxicity

has been observed. No other additional information is available at this time.

SIGNIFICANCE

The CSFs have significant potential for use in cancer patients. In this phase I trial, we hope to rapidly determine the effectiveness and maximally tolerated doses of GM-CSF so that we can rapidly enter them into trials to stimulate marrow recovery following combination chemotherapy administration.

PROPOSED COURSE

The trial will be completed as outlined, and the data thereby obtained will be used in the design of future studies.

PUBLICATIONS

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CM 09307-01 CRB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phase I Eval. of Tumor Necrosis Factor Plus IFN Gamma in Pts. with Solid Tumors

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

PI:	R. G. Steis	Chief	CRB, NCI
CoPI:	N. Rosen	Senior Investigator	MB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. Miller	Senior Staff Fellow	CRB, NCI
	S. Creekmore	Chief	BRB, NCI
	M. Lippman	Head	MBCS, NCI

COOPERATING UNITS *(if any)*
PRI, NCI-FCRF (Edward Crum and Walter Urba); Frederick Memorial Hospital (Margie Farrell); Genentech, San Francisco, CA (Steven Sherwin).

LAB/BRANCH
Clinical Research Branch

SECTION

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NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Clinical trials using single agent tumor necrosis factor have been ongoing for approximately the past 2 years. Minimal antitumor efficacy of this drug has been observed to date. In vitro studies with TNF, however, have demonstrated that the combination of gamma interferon with tumor necrosis factor results in significant augmentation of antitumor effects. These results have been confirmed in vivo in animal models. However, substantial enhancement of TNF-related toxicity has also been observed using this drug in combination with gamma interferon. In this study, we intend to investigate the immunomodulatory and toxic effects of the combination of TNF and gamma interferon.

To date, 9 patients have been admitted in this study at the lowest 3 dose levels. No antitumor effects have been observed. The results of immunologic evaluations of these patients is still in progress. Toxicity observed so far has included fever, chills, modest hypotension, nausea and vomiting, fatigue, and muscle soreness at the site of injection of the tumor necrosis factor but not at the site of injection the gamma interferon.

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Neal Rosen	Senior Investigator	MB	NCI
Dan L. Longo	Associate Director	OAD	NCI
Jeffrey Clark	Senior Staff Fellow	CRB	NCI
John W. Smith II	Senior Staff Fellow	CRB	NCI
Robin Miller	Senior Staff Fellow	CRB	NCI
Stephen Creekmore	Chief	BRB	NCI
Marc Lippman	Head	MBCS	NCI

OBJECTIVES

1. To determine the local and systemic toxicities of the combination of recombinant interferon gamma and TNF when administered intramuscularly every other day for 3 consecutive weeks.

2. To determine the maximally tolerated dose of TNF and gamma interferon on this treatment schedule.

3. To determine the effects of the combination of TNF and gamma interferon on immunologic functions including monocyte-related hydrogen peroxide generation and monocyte express of HLA-Dr and Fc receptors. In addition, the effects of the combination of the phagocytic activity of granulocytes will be determined.

METHODS EMPLOYED

Patients with solid tumors refractory to standard therapy are eligible for this study. Patients must have a performance status greater than 70 on the Karnofsky scale and have either measurable or evaluable disease. After an initial staging evaluation at the BRMP, groups of 3 patients will be admitted to increasing dose levels of both TNF and gamma interferon. There are 9 such dose levels ranging from a minimum of 10 micrograms/m^2 of TNF in combination with 10 micrograms/m^2 of gamma interferon up to a maximum of 100 micrograms/m^2 of TNF given in combination with 100 micrograms/m^2 of gamma interferon. If one of 3 patients at a given dose level develops Grade III toxicity, an additional 3 patients will be admitted to that dose level. If 2 of the 6 total patients at that dose level develop Grade III toxicity, higher dose levels of either TNF or gamma interferon will not be permitted.

Once maximally tolerated dose has been determined, this MTD would be administered to 6 patients concomitantly with aspirin at a dose of 650 mg p.o. qid during treatment days. Preclinical evaluations have shown that the toxicity of TNF given in combination with gamma interferon can be prevented by the concurrent administration of aspirin. In animal models this prevention of toxicity occurred without a concomitant decrease in antitumor efficacy.

Immunologic monitoring will include those tests mentioned previously.

MAJOR FINDINGS

So far 9 patients have been admitted to the study, 3 at each of the lowest 3 dose levels. Toxicities so far have included the development of pain at the site of the TNF injection (but not at the site of the gamma interferon injection), fever, chills, nausea, vomiting, fatigue, and modest hypotension. No patient has yet required discontinuation of therapy. No antitumor effects have been observed so far and the immunologic data is only now being collected.

SIGNIFICANCE

Tumor necrosis factor has been in clinical trials for 2 years, and no significant antitumor effects have been observed. This compound initially demonstrated promising in vitro effects against tumor but not normal cells and promising in vivo effects in murine tumor systems. It was hoped that by combining gamma interferon with tumor necrosis factor that greater antitumor effects might be observed in patients. In this study we hope to define maximally tolerated doses and maximal biological response modifying doses of the combination of these drugs.

PROPOSED COURSE

Further patients will be admitted to this study in order to determine maximally tolerated doses and optimal biological response modifying doses.

PUBLICATIONS

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 09308-01 CRB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Efficacy Study of Recombinant Leukocyte A IFN in Hairy Cell Leukemia

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

PI:	R. G. Steis	Chief	CRB, NCI
Others:	D. L. Longo	Associate Director	OAD, NCI
	J. W. Clark	Senior Staff Fellow	CRB, NCI
	J. W. Smith II	Senior Staff Fellow	CRB, NCI
	R. L. Miller	Senior Staff Fellow	CRB, NCI
	S. P. Creekmore	Chief	BRB, NCI

COOPERATING UNITS *(if any)*

PRI, NCI-FCRF (Walter Urba and Edward Crum); Frederick Memorial Hospital (Carolyn Schoenberger)

LAB/BRANCH

Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This study is an old but ongoing study evaluating the efficacy of recombinant alpha interferon in patients with hairy cell leukemia. Fifty-six patients have been admitted to this study and of these 43 continue on therapy. The overall initial response rate was 92 percent of 54 patients evaluable for response. There have been 2 complete responses, 33 partial responses, 15 minor responses, and 4 patients did not respond. Two patients are not evaluable for hematologic response. One received only a single dose of interferon prior to the discovery of a malignant glioma, and the second patient is not evaluable for hematologic response because she was treated for painful bony involvement proven to be due to hairy cells. One patient who did not respond at 3 million units daily was dose escalated to 6 million units daily and subsequently had a minor response which is ongoing. Seven patients who responded initially ultimately had disease progression. Six of these were treated with higher doses of interferon and 3 have responded. The response in these 3 patients however has been limited and transient. Forty-three responding patients continue to respond.

Toxicity has been minimal and acceptable. The most common side effect has been minor fatigue which in no way has been limiting.

GPO 914-918

PROJECT DESCRIPTION

PERSONNEL

Ronald G. Steis	Chief	CRB	NCI
Dan L. Longo	Associate Director	OAD	NCI
Jeffrey W. Clark	Senior Staff Fellow	CRB	NCI
John W. Smith II	Senior Staff Fellow	CRB	NCI
Robin L. Miller	Senior Staff Fellow	CRB	NCI
Stephen P. Creekmore	Chief	BRB	NCI

OBJECTIVES

To evaluate the safety and efficacy of recombinant interferon alpha in patients with hairy cell leukemia.

METHODS EMPLOYED

Patients to be considered eligible for this study must have histologically documented hairy cell leukemia and one or more cytopenias. At the initiation of therapy, all patients must have a platelet count of less than 100,000 per cubic millimeter and/or a granulocyte count of less than 1,500 per cubic millimeter and/or a hemoglobin of less than 10 grams percent or transfusion dependency. After their initial clinical evaluation, patients are then given 3 million units of recombinant alpha interferon subcutaneously daily for 4 to 6 months. In responding patients, maintenance therapy is given at a dose of 3 million units subcutaneously 3 times per week. Responding patients have continued on therapy indefinitely. Patients who might experience disease progression are given escalated doses of recombinant alpha interferon in an attempt to control their disease. If dose-limiting side effects develop at the higher doses, patients are taken off study.

MAJOR FINDING

The overall response rate has been 92 percent with 2 complete responses, 33 partial responses, 15 minor responses, and 4 non-responders among 54 evaluable patients. Two patients are not evaluable for response. One of these non-evaluable patients had a second neoplasm discovered shortly after starting interferon that required intervention and 1 was treated with essentially normal peripheral blood indices because of the development of painful bony infiltration with hairy cells. One of the non-responders at the initial dose of interferon was dose escalated to 6 million units a day and is now a minor responder. His response is ongoing. Seven patients ultimately developed progressive disease despite continued administration of interferon and 6 were given higher doses of interferon. Three of these 6 patients have now responded although to a minor degree and transiently. Toxicity has been minimal and has consisted of at most minor fatigue.

SIGNIFICANCE

This study confirms the significant activity of recombinant alpha interferon in patients with hairy cell leukemia. The problems that have developed in these

patients include the development of disease resistance to interferon in 7 of the 50 initially responding patients. The mechanism of this resistance is not known at present. However, a number of our patients have developed neutralizing antibodies to alpha interferon, and these may mediate the apparent tumor-cell resistance to this drug.

PROPOSED COURSE

Responding patients are continuing on alpha interferon because the vast majority of patients continue to do well but with less than a complete response to therapy. These patients will be offered entry to a proposed new study in which either partial or minor responses will be stratified according to response and then randomized to either continued interferon for 3 further months or to deoxycoformycin for a total of 6 doses. In our hands deoxycoformycin has resulted in significant responses in hairy cell patients resistant to prior interferon therapy. We hope to use deoxycoformycin to convert partial and minor responders to complete responders.