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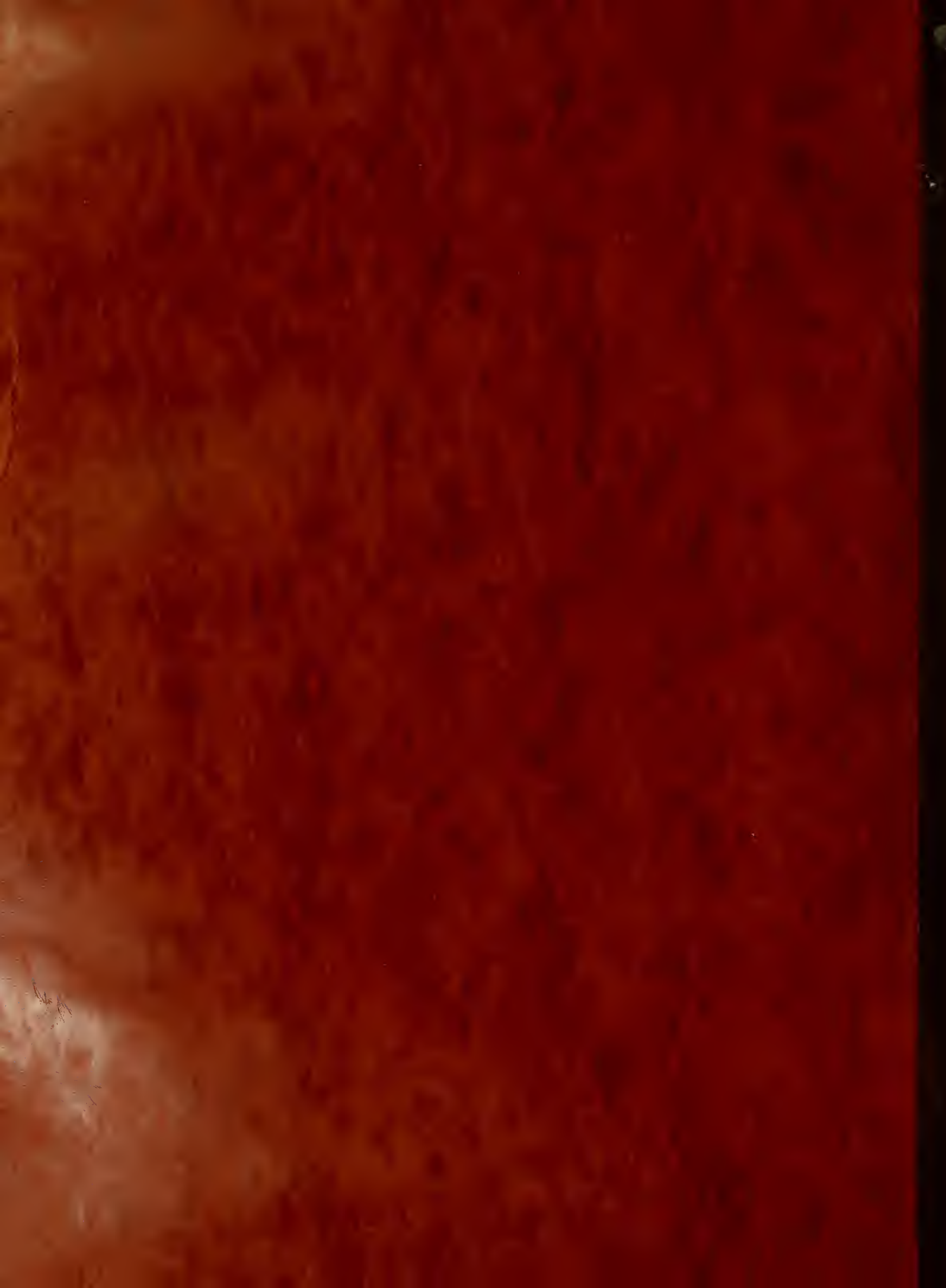
Center for Biologics Evaluation and Research
Division of Hematology

Annual report
1991-1992



Center for Biologics Evaluation and Research (U.S.) annual report

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October 1, 1991 to September 30, 1992

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ANNUAL REPORT - 1992

Division of Hematology

REPORT OF THE DIVISION DIRECTOR

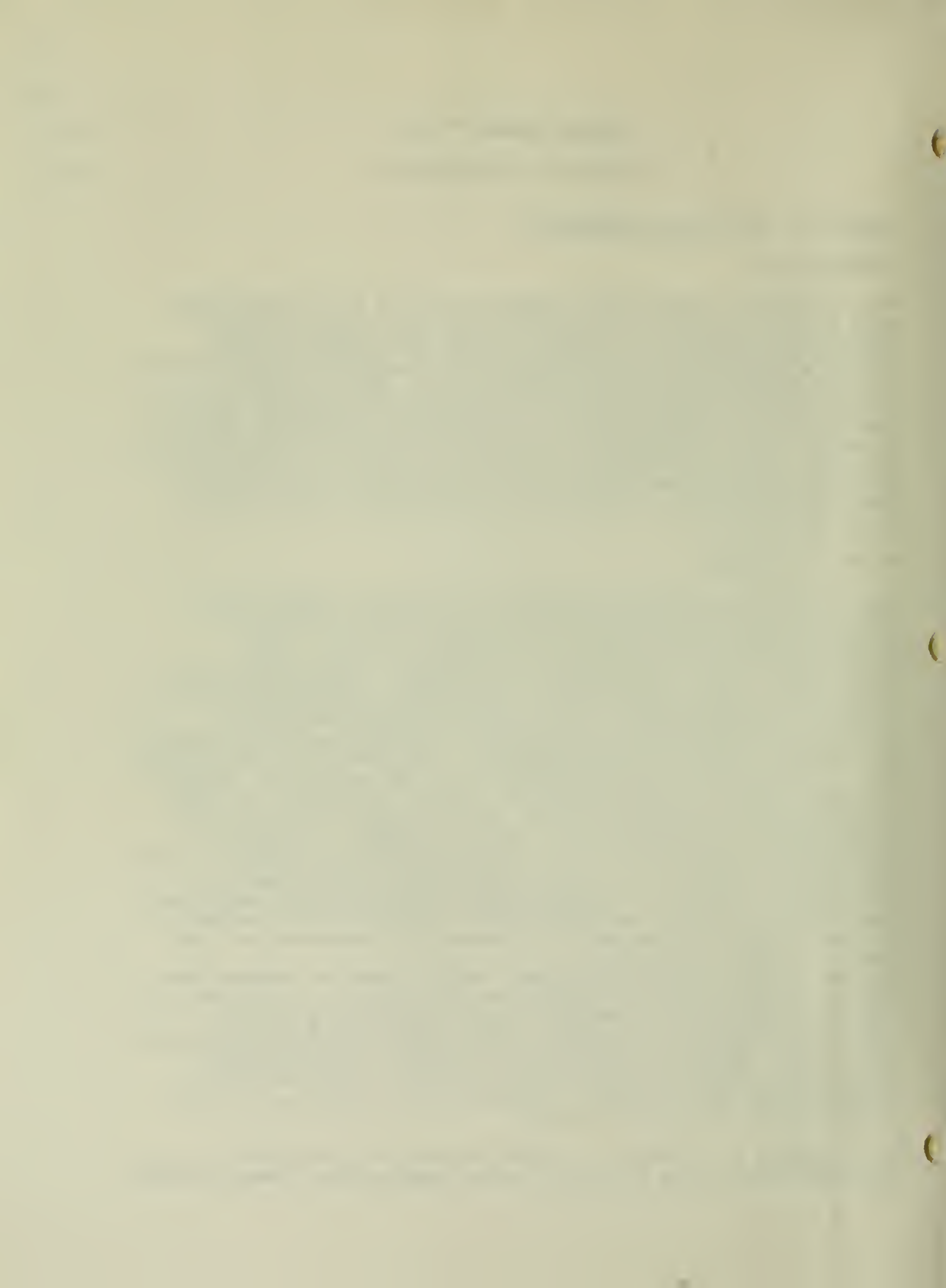
INTRODUCTION

The Division of Hematology comprises four of the laboratories that existed with the Division of Blood and Blood Products, namely, the Laboratory of Cell Biology, The Laboratory of Cellular Hematology, the Laboratory of Hemostasis and Thrombosis, and the Laboratory of Plasma Derivatives. Each of these functions under the guidance of a permanent Laboratory Chief. The Division of Hematology has operated with an Acting Division Director (who also serves as the Divison's Associate Director for Science) and an Acting Deputy Division Director. A permanent Division Director was appointed on September 25, 1992. The responsibilities of the Division are described in the Mission Statement (below).

MISSION STATEMENT

The Divison of Hematology, Office of Biologics Research has responsibility for scientific programs involved with the evaluation of biologic products related to blood. This evaluation of biologics deals with aspects such as their preparation (e.g., manufacture), properties (characterization), mechanism of action, measurement (analysis), stability, clinical effectiveness and safety. The products themselves include materials isolated from human blood, analogous materials prepared by the use of other technologies (e.g., hybridoma or recombinant DNA), and still others prepared from a wide variety of sources (animal, tissue culture, microbial, plant, synthetic) but used for analogous clinical purposes. Such clinical uses span the prophylaxis and treatment of bleeding disorders, thrombotic conditions, infectious diseases, immunodeficiency, anemia, shock, and graft rejection, as well as the diagnosis (in vivo) of cardiologic, neoplastic, hematologic, and infectious conditions. The Division is also responsible for evaluation of a variety of devices, such as those used in preparation, preservation, and storge of blood products.
As part of the evaluation process, and in order to recruit and retain skilled scientific reviewers, the Division initiates and conducts laboratory investigations of biological problems concerning coagulation factors, fibrinolytic enzymes, proteolytic inhibitors, immunoglobulins (both monoclonal and polyclonal), cells of the immune and hemostatic systems, oxygen-carrying compounds, plasma proteins, erythropoietic hormones, and viral contamination of blood products.

On the basis of information gained through the activities described above, the Division reviews applications, based largely



on laboratory and clinical data, and makes recommendations for or against approval. The procedures for review may involve presentations to Advisory Committees, conferences with manufacturers, and, on occasion, meetings of broader scope such as workshops and symposia. Thus, the Division serves as the primary source within the Agency for scientific information and recommendations about products in its area of responsibility.

In parallel with these laboratory investigations and review activities, the Division develops regulatory policies and documents for products within its purview and maintains liaisons with other parts of the FDA, other government agencies, regulatory components of foreign governments and international bodies, academic institutions, and non-governmental organizations representing manufacturers or consumers.

RESEARCH ACTIVITIES

Research conducted by Division scientist resulted in 47 communications that have been published or are in press, as well as a number of others that have been submitted for publication. In addition, more than 30 presentations were made at local, national, and international meetings.

The following represent some of the year's major research contributions by the Divison of Hematology.

Laboratory of Cell Biology

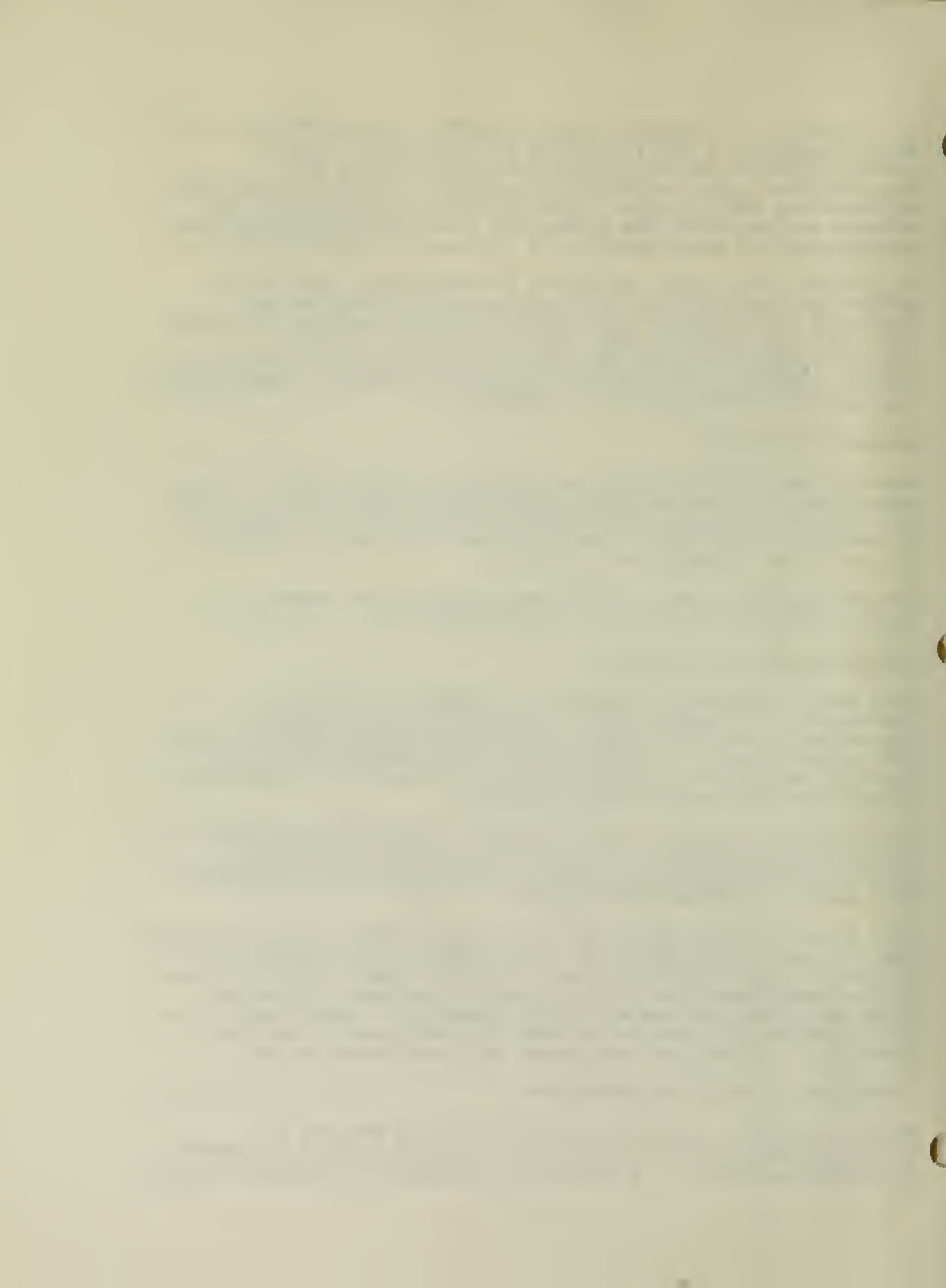
Work has progressed on biochemical pathways of signal transduction in immune cells, in particular the coupling mechanism between the T cell receptor/CD3 complex of lymphocytes and phospholipase C. Perturbation of the complex with anti-receptor antibodies and observation of consequent cytoskeletal changes have yielded promising results.

B and T cell immunity in HIV infection, using Brucella abortus as a probe, was studied. Conditions for conjugating *Brucella abortus* lipoplysaccharide to HIV-1 components so as to achieve a more useful immunogen were explored.

SCID mice were used as a model for immunotherapy of HIV infection and stem cell reconstitution, while homeotic gene expression and regulation were studied in immune cells. Studies also continued on the regulation of multiple monocyte functions, including cytokine generation and role of Fc receptors. Specifically, the participation of monocyte inflammatory mediators in adverse reactions to monoclonal antibodies has been investigated.

LABORATORY OF CELLULAR HEMATOLOGY

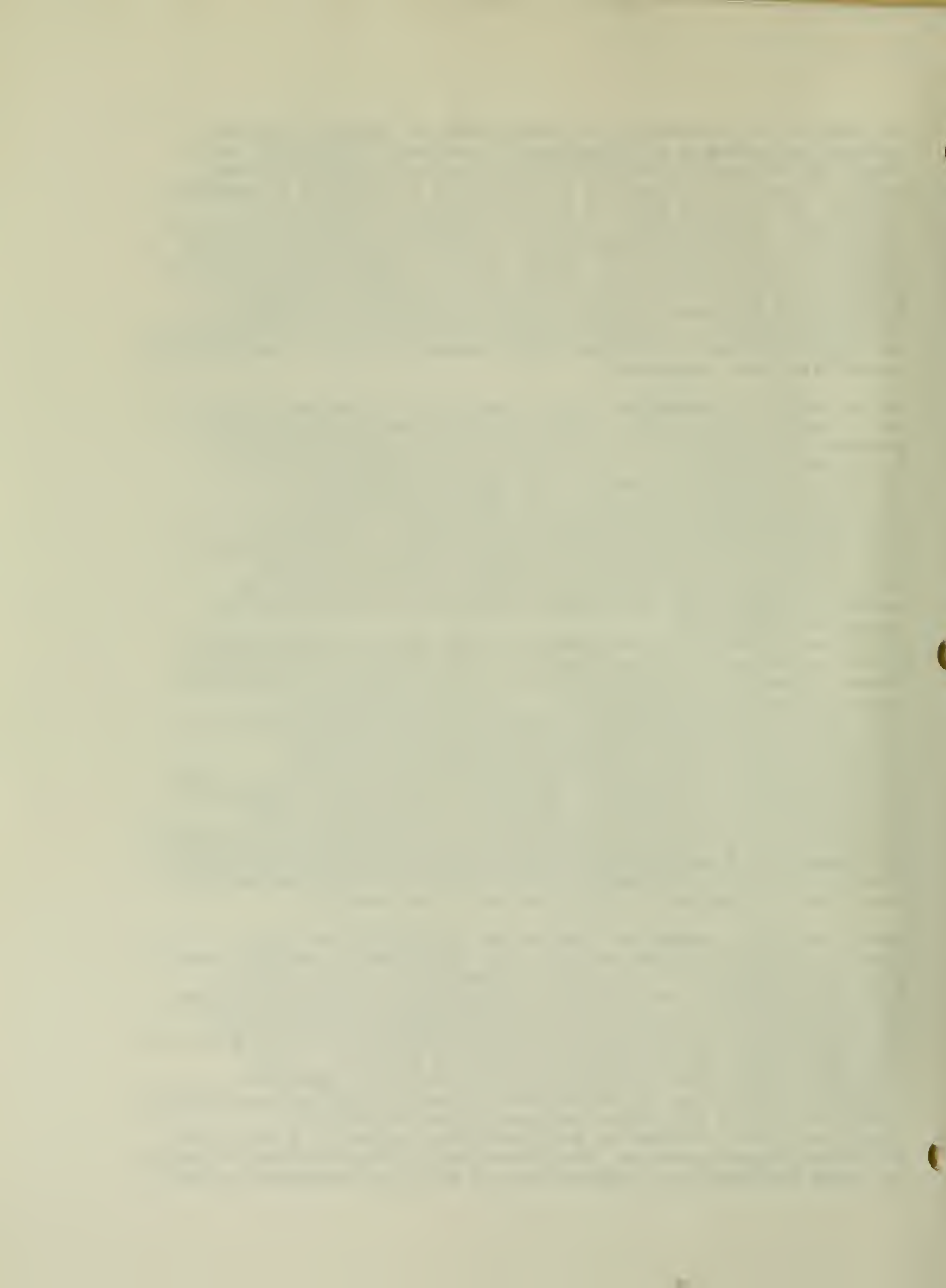
Work on platelet function has continued with the use of techniques such as flow cytometry and the microplate procedures developed previously. Microvesicle formation, a phenomenon known



to occur during storage, has been found to involve calpain-induced proteolysis of cytoskeletal proteins, including actin. Since the elusive "platelet storage lesion" exerts a global effect on cell function, it is likely to be related to membrane effects and studies on cell signal transduction have been initiated. Specifically, work is underway to study the role of transmembrane calcium flux to the problems related to storage. Studies of the inhibitory effects of beta lactam antibiotics on platelets demonstrated that penicillin G induces platelet dysfunction by impairing the activation-linked expression of surface receptors. Projects directed toward an understanding of the changes in platelet reactivity induced by viral inactivation procedures were completed.

In the work on leukocytes, there was study of the mechanisms by which neutrophils migrate toward chemical attractants. This process (chemotaxis) is a critical host defense mechanism involved in infectious, immunologic and neoplastic disorders. In order to better understand the basic biochemical, physiological and structural requirements for leukocyte migration, several approacheshave been taken, including biochemical assays of cytoskeletal F-actin, fluorescent probe studies with flow cytometry and laser scanning confocal microscopy, and physiological studies directly measuring leukocyte migration, are used to dissect the mechanisms of leukocyte chemotaxis.
This year a series of CD45 negative RAW 264 macrophage mutant clones were generated and found to have defective chemotaxis. These clones will provide a useful set of tools to explore the molecular basis of chemotaxis. A project which has systematically evaluated the adhesion molecule expression on adherent and nonadherent human neutrophils exposed to chemoattractant, has provided new information on the down-regulation of adhesion molecules which is associated with chemotactic responsiveness. The down regulation of adhesion molecules during neutrophil chemotaxis, using monoclonal antibodies to an array of these proteins has also been explored. The observation has been made that there are distinct patterns of adhesion molecule expression correlating with the chemotactic responses of distinct subpopulations of neutrophils.

Among the major questions that arise in connection with hemoglobin-based oxygen carriers for therapeutic use are those involving toxicity. It is now agreed that the endothelial relaxing factor, a vasodilator, is primarily nitric oxide (NO). The interactions between NO and human hemoglobin have been studied and it was found that cross-linking changes the alpha and beta chain heme pockets and the rates of binding. This observation may have important relationships to observed hemoglobin toxicity, at least some of which seems to result from vasoconstrictive effects of extracellular hemoglobin.
There were also studies on modified hemoglobins as a source of activated oxygen species, the formation of altered heme products by human hemoglobins, interactions of modified hemoglobins with



iron chelators and effects of hypothermic conditions on the oxygen carrying capacity of crosslinked hemoglobins.

Laboratory of Hemostasis and Thrombosis

Immunochemical studies were focused on development and use of antibodies which neutralize the various activities of tissue factor pathway inhibitor (TFPI). This inhibitor is a circulating plasma protein that binds to coagulation factor Xa and inhibits Factor Xa activity.

The relationship between fibrinolytic activity and metastatic potential in three osteosarcoma cell lines is under investigation. The cell lines appear to have different levels of plasminigen activator inhibitor and urokinase plasminogen activator expression. The details of these relationships will continue to be explored.

Epitope mapping of human antibodies to factor VIII and von Willebrand factor (vWF) continue. Thrombin digestion of factor VIII has yielded data on material from two patients, while the techniques for trypsin digestion of F VII and vWF have been optimized. Immunoprecipitation experiments are being planned.

The Seroconversion Surveillance Project, a collaborative study designed to monitor users of clotting factor concentrates for evidence of transmission of HIV has investigated 44 conversions since 1987. Nine of these reflect probable HIV serocenversion due to virus-inactivated concentrate, but none of these products are on the market at this time.

Laboratory of Plasma Derivatives

There was continued progress in physical and chemical characterization of plasma derivatives. The previously developed themodynamic model for ligand-induced protein denaturation (the practical application is the albumin-stabilizer interaction) was extended to explain the number of denaturation peaks that occurs. During the past year, the studies involved computer modeling and utilized a differential scanning calorimeter, which was also calibrated and validated, in addition to a photodiode array spectrophotometer.

The attempt to design effective vaccines that do not utilize the infectious agent continued. Because the antiidiotype approach to the production of an antiflavivirus vaccine was not successful, the peptide representing the protective epitope on flavivirus E-glycoprotein will be sought, in hopes that this may be employed as a subunit vaccine effective against members of the flavivirus family.

The partitioning of hepatitis C virus during plasma fractionation was studied using an assortment of techniques, including reverse

transcriptase activity and polymerase chain reaction. Fractionation of a plasma pool derived solely from anti-HCV reative donations showed that the virus partitioned mostly into cryoprecipitate, fraction I and fraction III, with a trace amount in fraction II. It was calculated that the fractionation process leading to immune globulin resulted in an overall reduction in HCV RNA by a factor of 47,000.

REGULATORY ACTIVITIES

The Laboratory of Cell Biology continued to handle a heavy load of applications dealing with imaging and hematologic applications of monoclonal antibodies.

The Laboratory of Cellular Hematology, whose responsibilities span a wide range of products related to the cellular elements of blood, reviewed some 400 IND, NDA and 510(K) submissions, as well as more than 100 license applications and amendments.

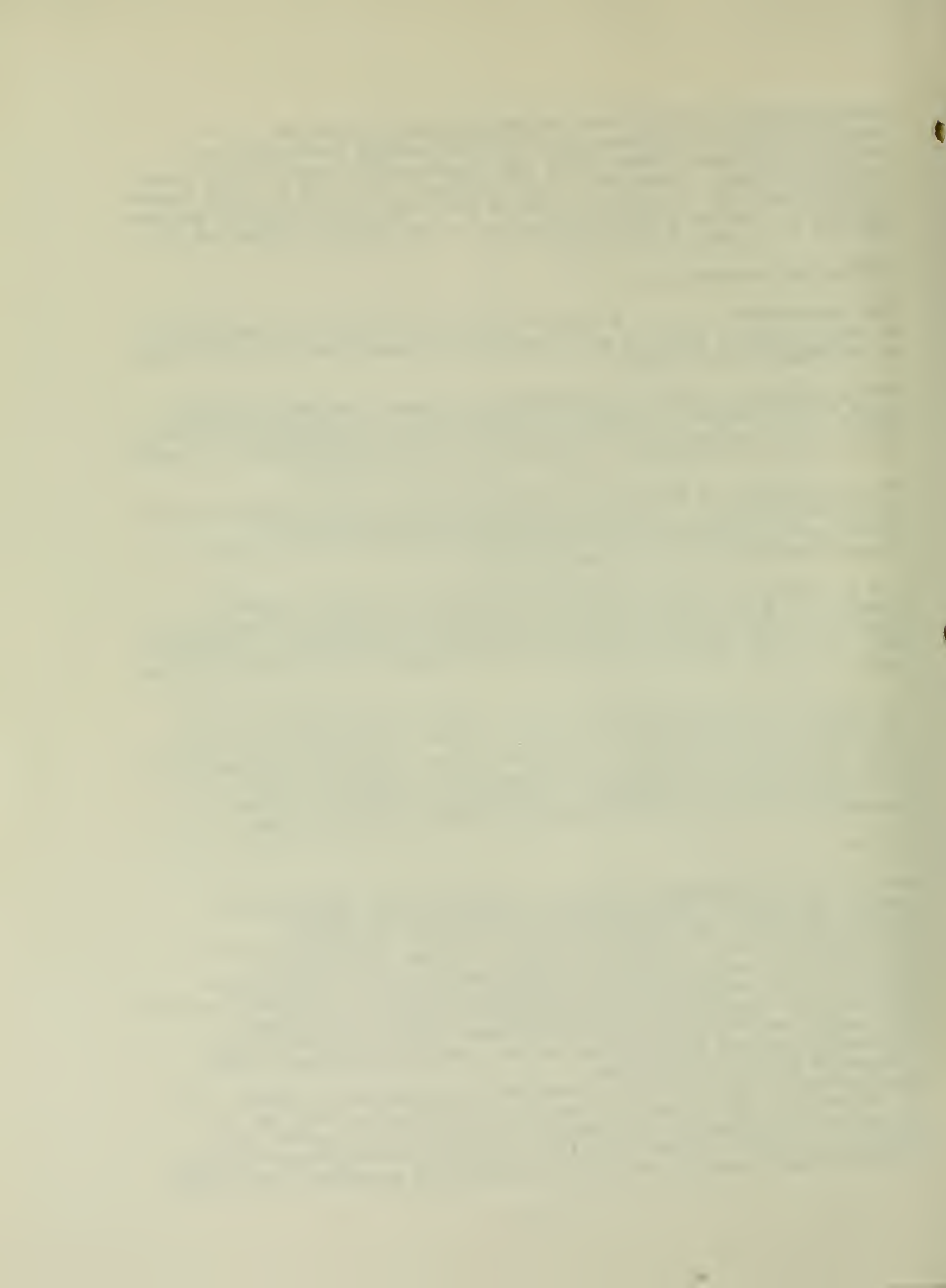
The Laboratory of Hemostasis and Thrombosis, with responsibility for procoagulants, fibrinolytic agents and proteolytic inhibitors, reviewed over 300 IND submissions and over 100 license applications and amendments.

The Laboratory of Plasma Derivatives, which handles immune globulins, proteolytic inhibitors derived from human plasma and plasma volume expanders (plasma-derived and synthetic), reviewed more than 200 IND submissions and took final action on 36 license applications.

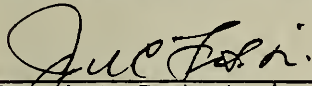
The numbers listed above are only a partial indication of the regulatory work performed by the Division, whose members spent many hours with personnel from CBER, from elsewhere in FDA, from other government agencies and from provate organizations developing product standards, determining points of scientific consensus and developing regulatory policy and positions.

FUTURE

During the forthcoming year, the Division will experience a number of administrative changes related to the CBER reorganization. Anticipated events are the relocation of the Laboratory of Cell Biology and the transfer of regulatory authority for some products (erythropoietin and thrombolytic agents). The availability of additional space (contingent on the movement of the Division of Transfusion Science to larger quarters), and the hiring of additional staff (dependent upon CBER ceilings and the effects of user fee legislation) could favorable affect activities.
Most research projects will continue, and some will expand. It is hoped that the growth of the research program will be in directions that permit the staff to broaden and improve their skills. In view of the fact that the Division's responsibility includes both traditional and biotechnology products, its scope

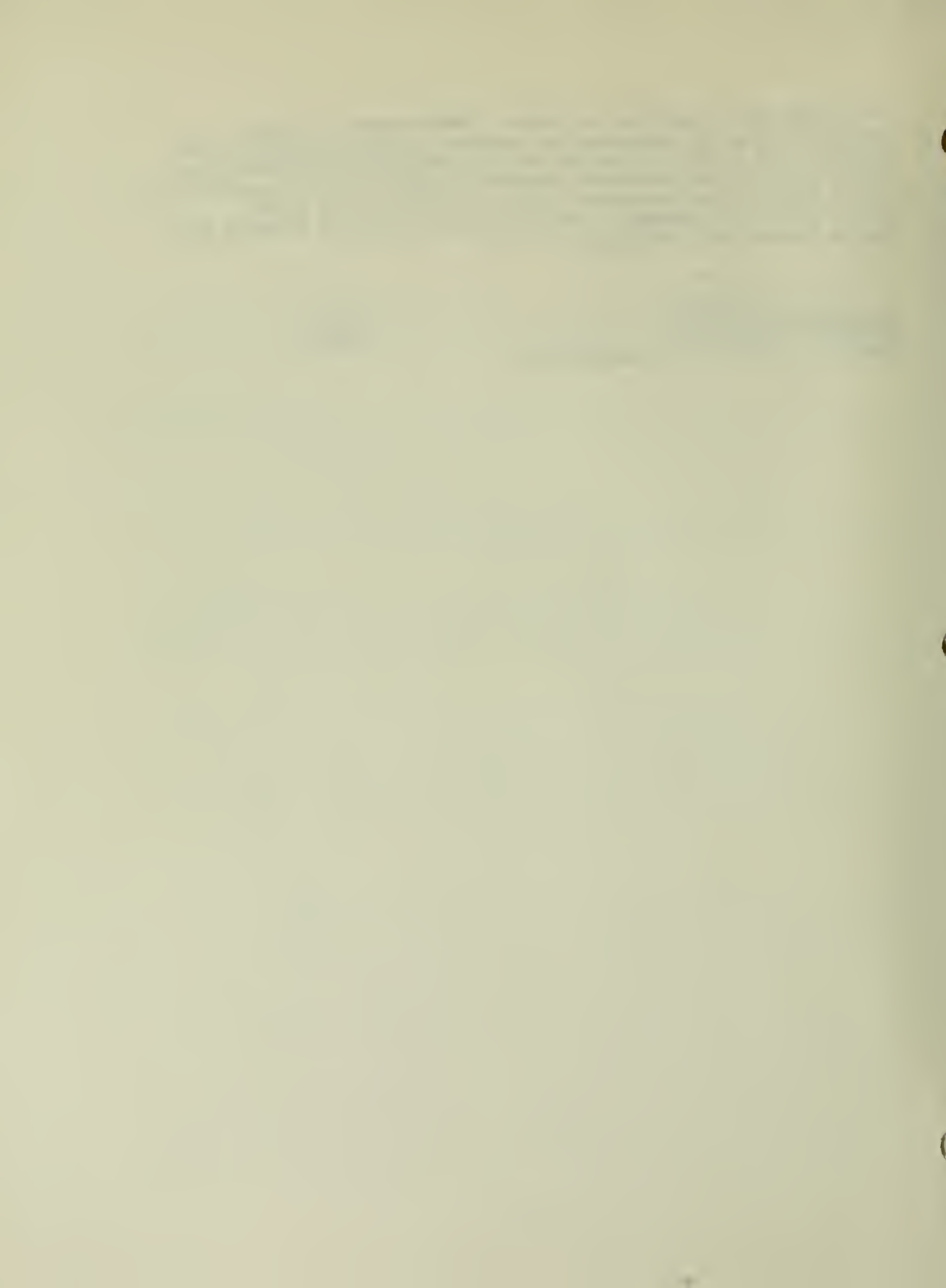


and vision must continue to widen. The pressure of the regulatory load is expected to increase as more products move from the IND to the PLA stage, as new manufacturers enter the field, and as manufacturers respond to CBER's encouragement to effect product improvements (e.g., more effective viral inactivation procedures). Meeting these multiple demands will pose a persistent challenge.



Joseph C. Fratantoni
Director, Division of Hematology

10/26/92
Date



Division of Hematology

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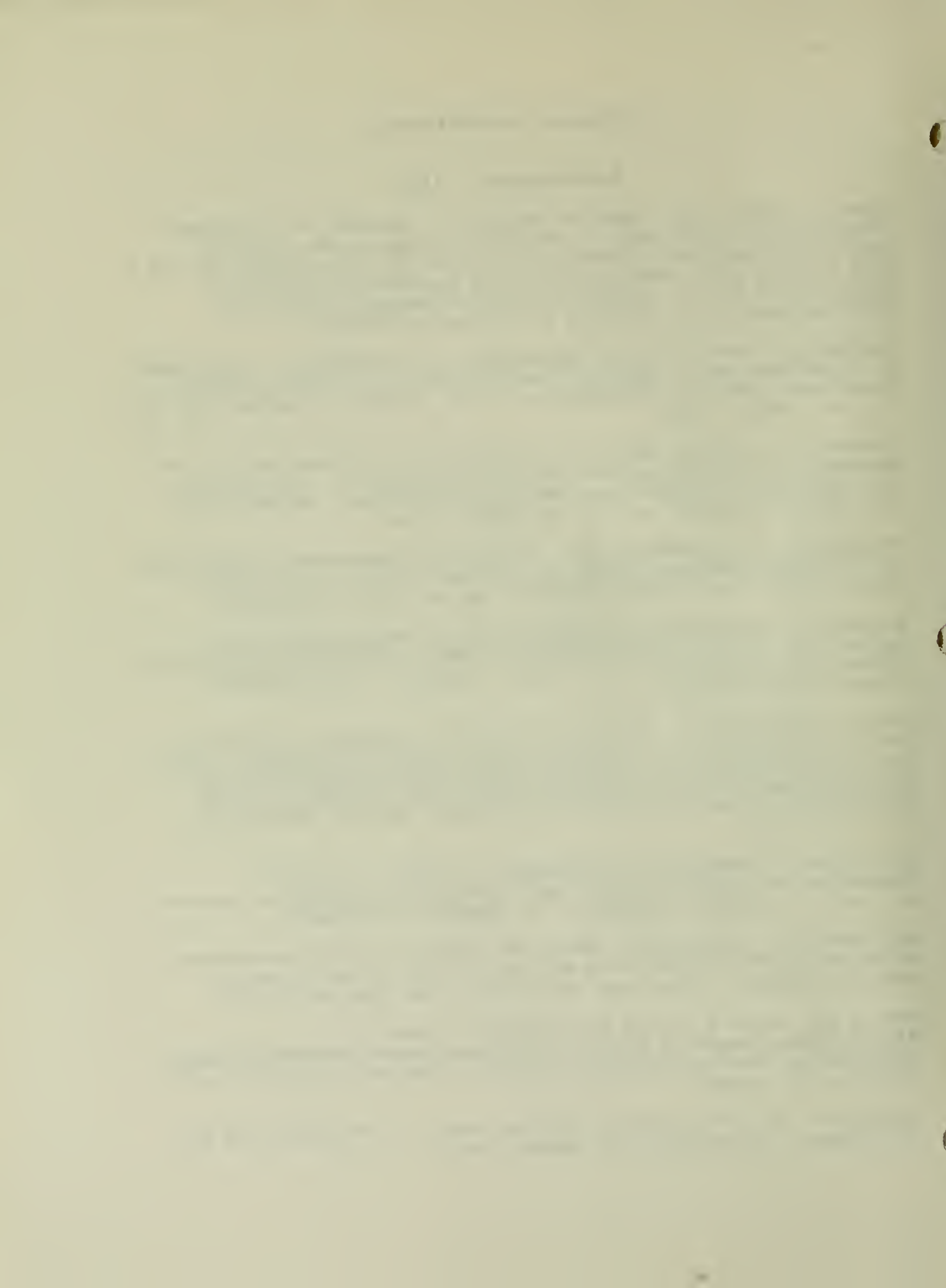

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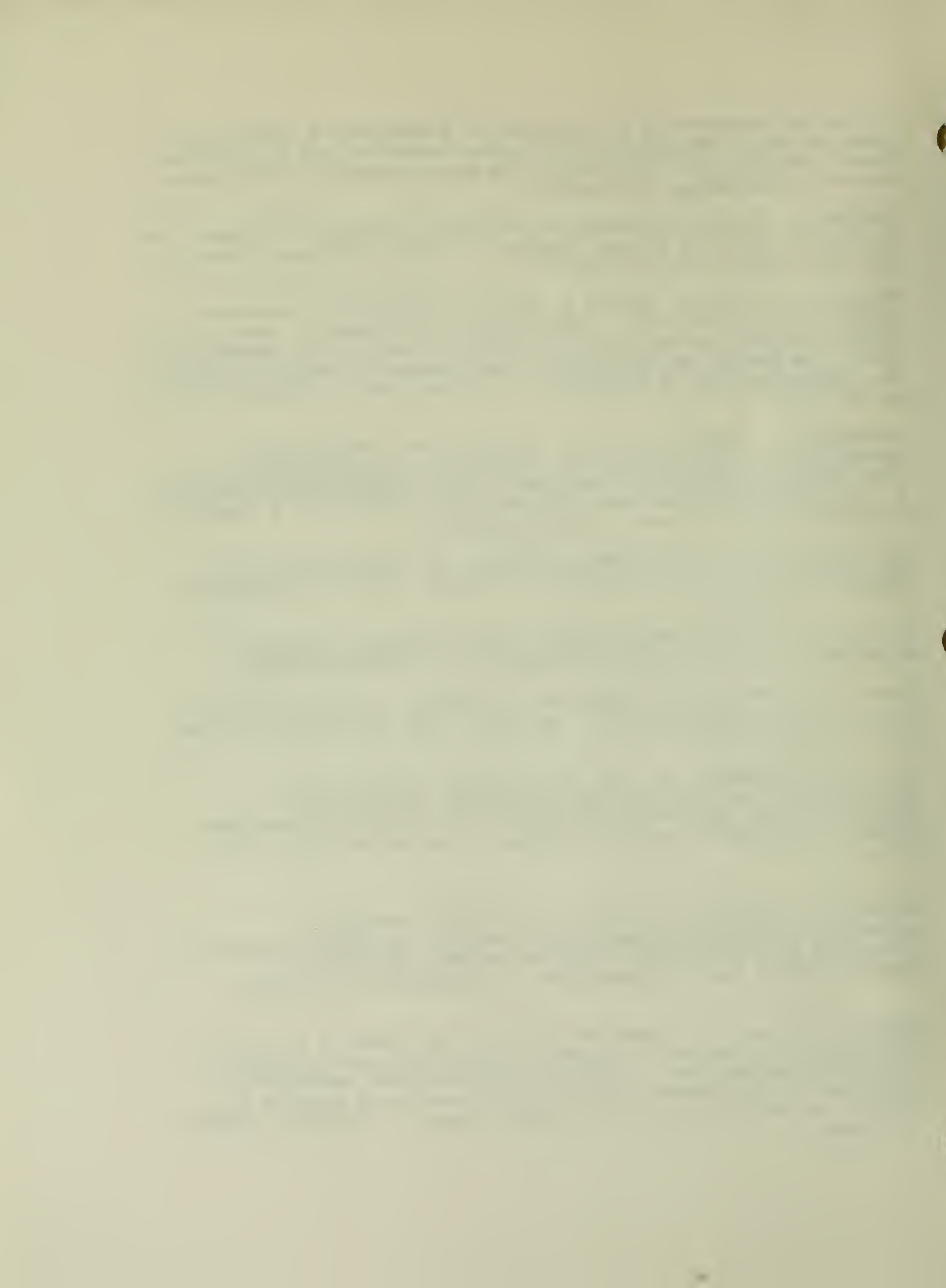

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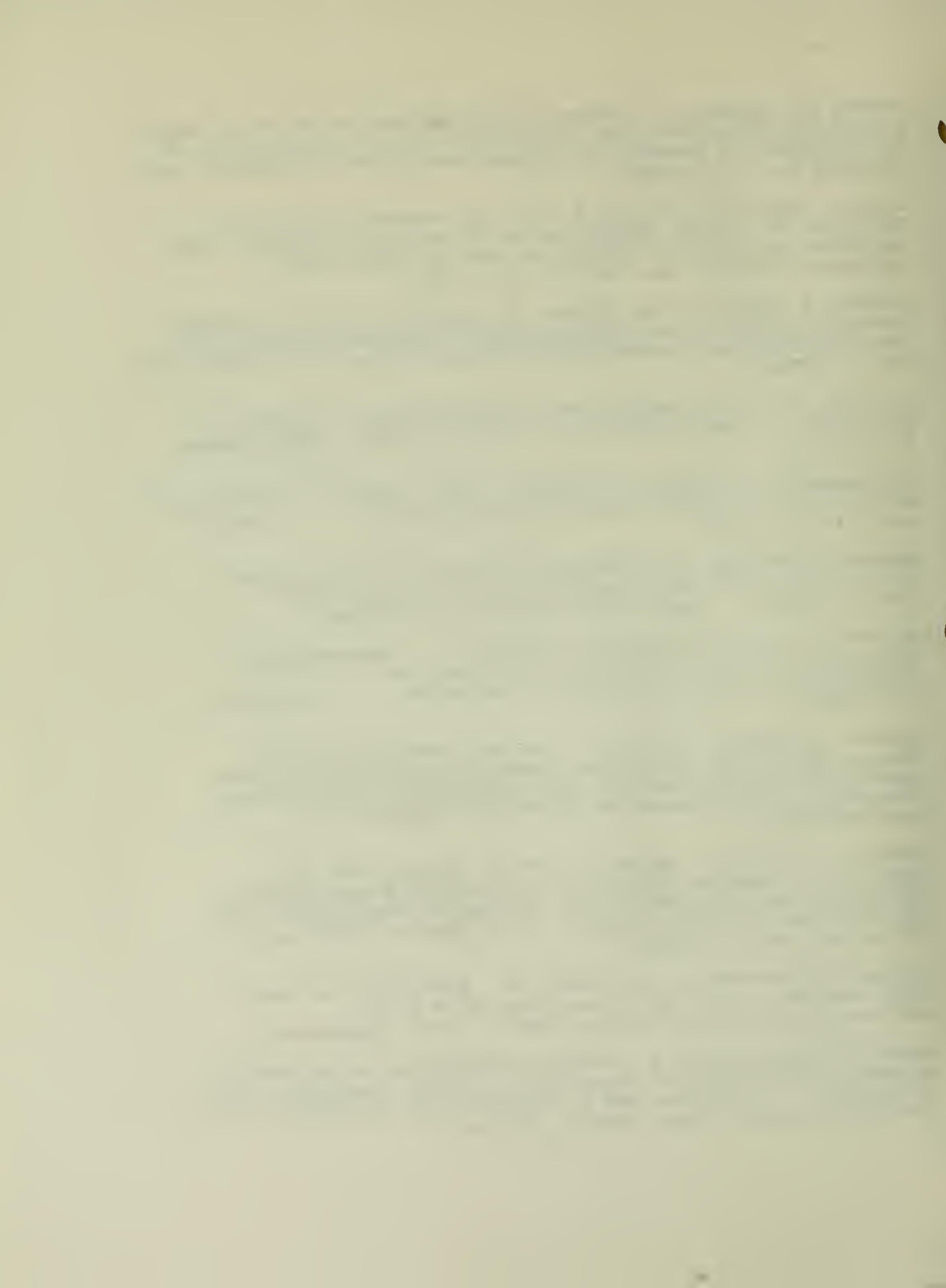

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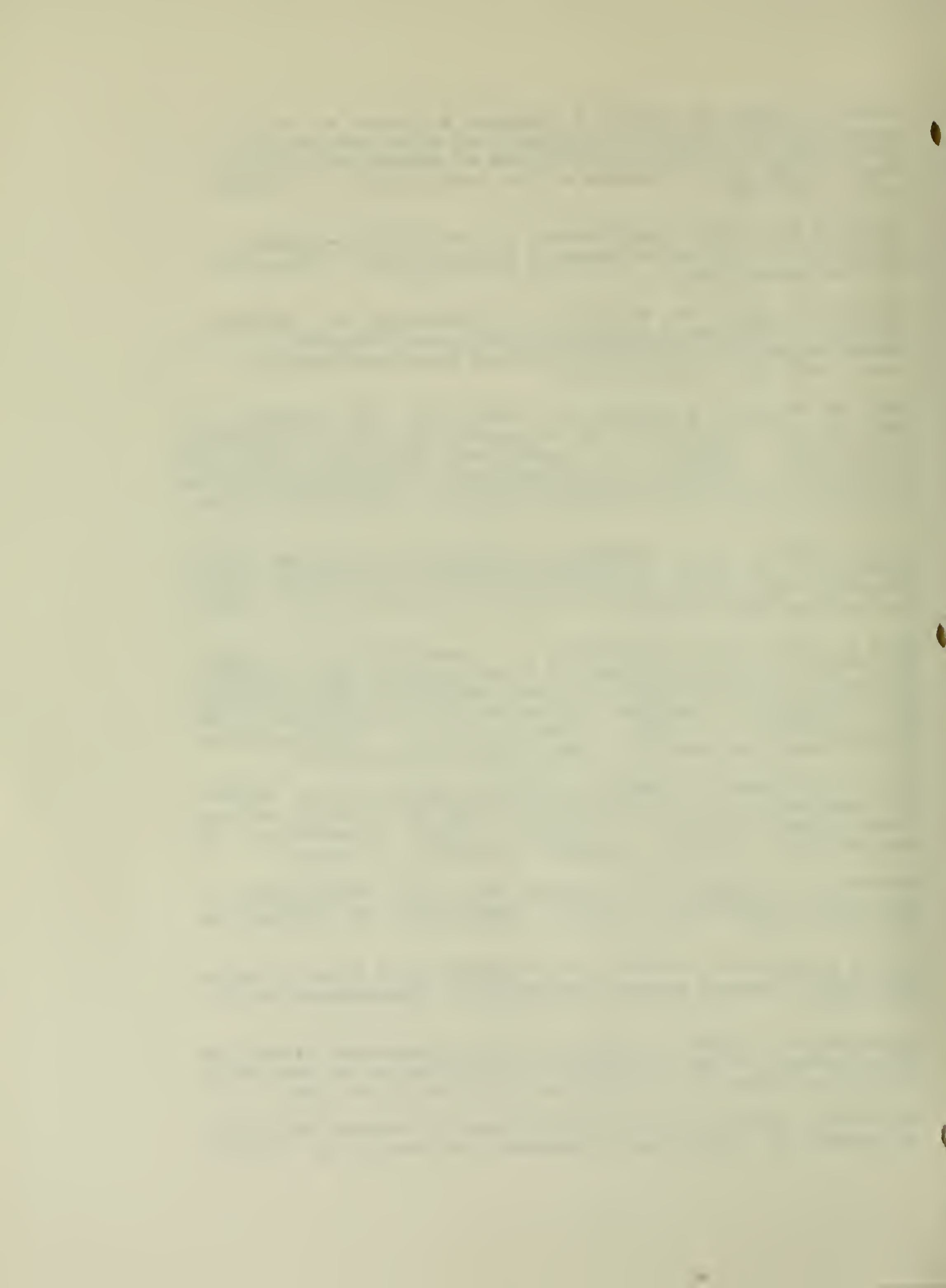

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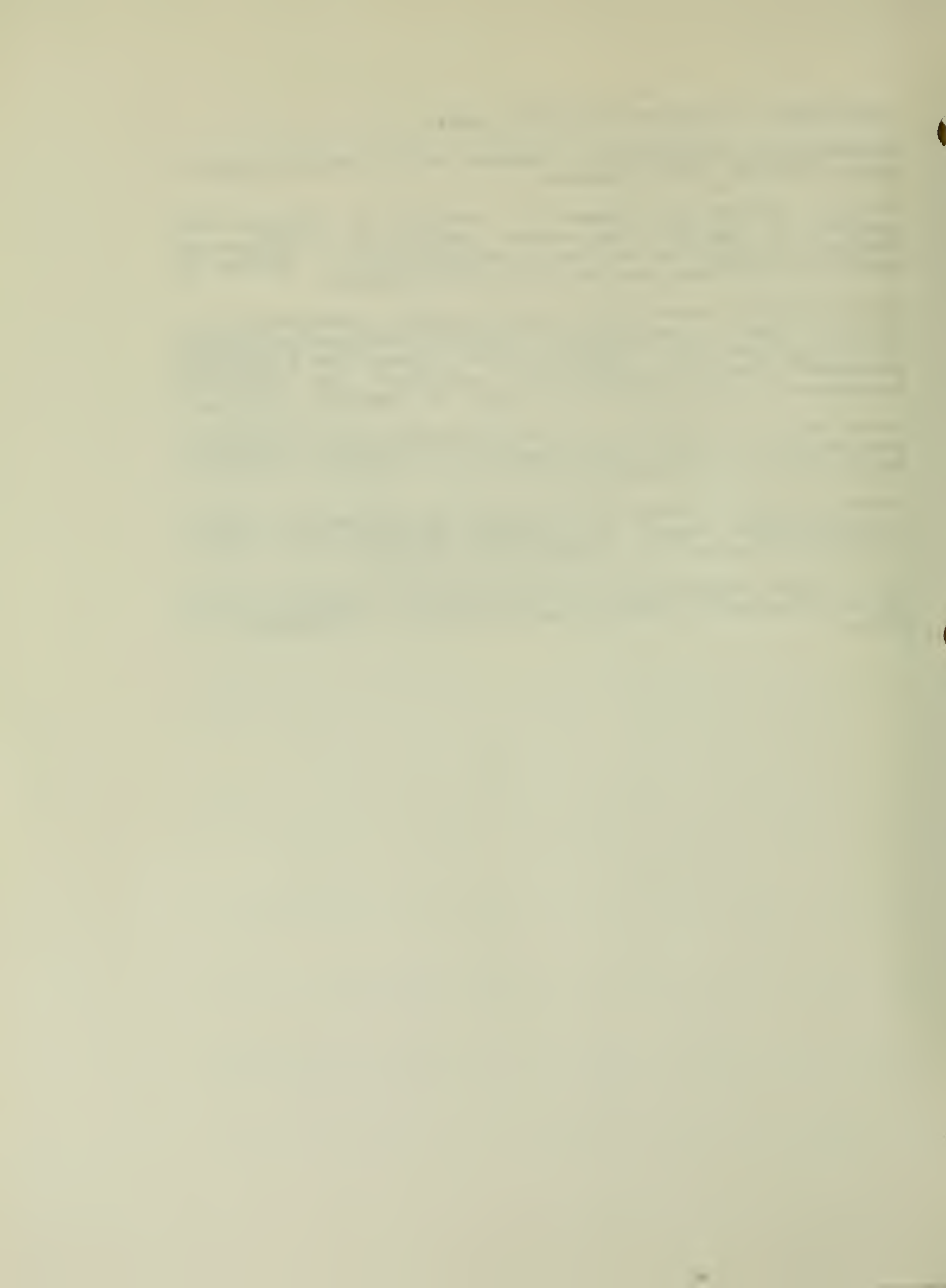

Annual Report

Laboratory of Cell Biology

1992

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Senior Scientist	John Jessop, Ph.D.
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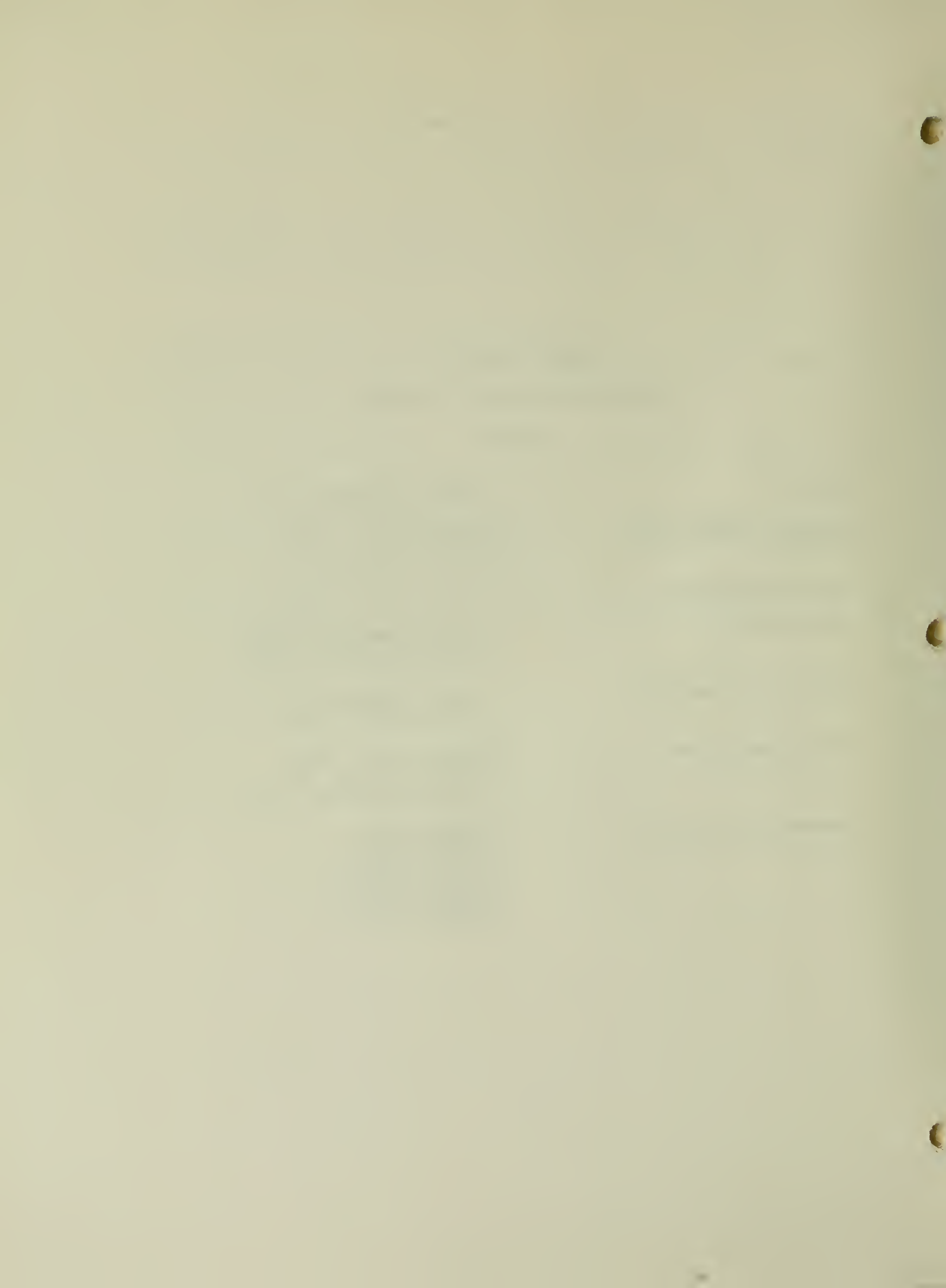
Visiting Fellow	Maria Alava, Ph.D. Antonio Conti, Ph.D. Julia Goldstein, M.D.
Research Assistant	Michael Betts Karen Debell Sylvia Johnson Young Lim Lee Elaine Lizzio



OVERVIEW:

The Laboratory of Cell Biology is responsible for the regulation of monoclonal antibodies intended for *in vivo* use, as diagnostic or therapeutic modalities. During the period of this report, over 40 original Investigational New Drug (IND) submissions were reviewed by the medical senior staff, raising the total number of submissions under consideration to over 400. In addition, over 600 supplements were reviewed. Four Product License Applications (PLA's) for monoclonal antibodies were in the process of review with members of the LCB serving as chairs. Staff also served on three additional committees. Currently, seven PLA's are under review: an anti-melanoma imaging agent, an anti-myosin imaging agent for diagnosis of myocardial injury, two anti-CEA's for imaging of colon cancer, an anti-CD5 (T cell) for treatment of graft-versus-host disease, and two IgM anti-LPS (one murine one human) for treatment of gram-negative sepsis.

In keeping with the philosophy of the laboratory, all staff members were actively involved in original basic research in Immunology/Cell Biology. The areas of investigation included experimental use of monoclonal antibodies and studies of the biochemical consequences of stimulation of the surface molecules by monoclonal antibodies. In addition, fundamental studies of effector function of immune cells (monocytes and T & B cells) and their modulation by lymphokines were carried out. As part of the laboratory's commitment to AIDS research, studies on the nature of the antibody response to HIV infection were initiated. Significant advances resulting in publications in peer reviewed journals (see bibliography attached) and presentations at scientific meetings were achieved.

Specific projects:

1. Biochemical pathways of signal transduction in immune cells; monoclonal anti-receptor antibodies as probes.

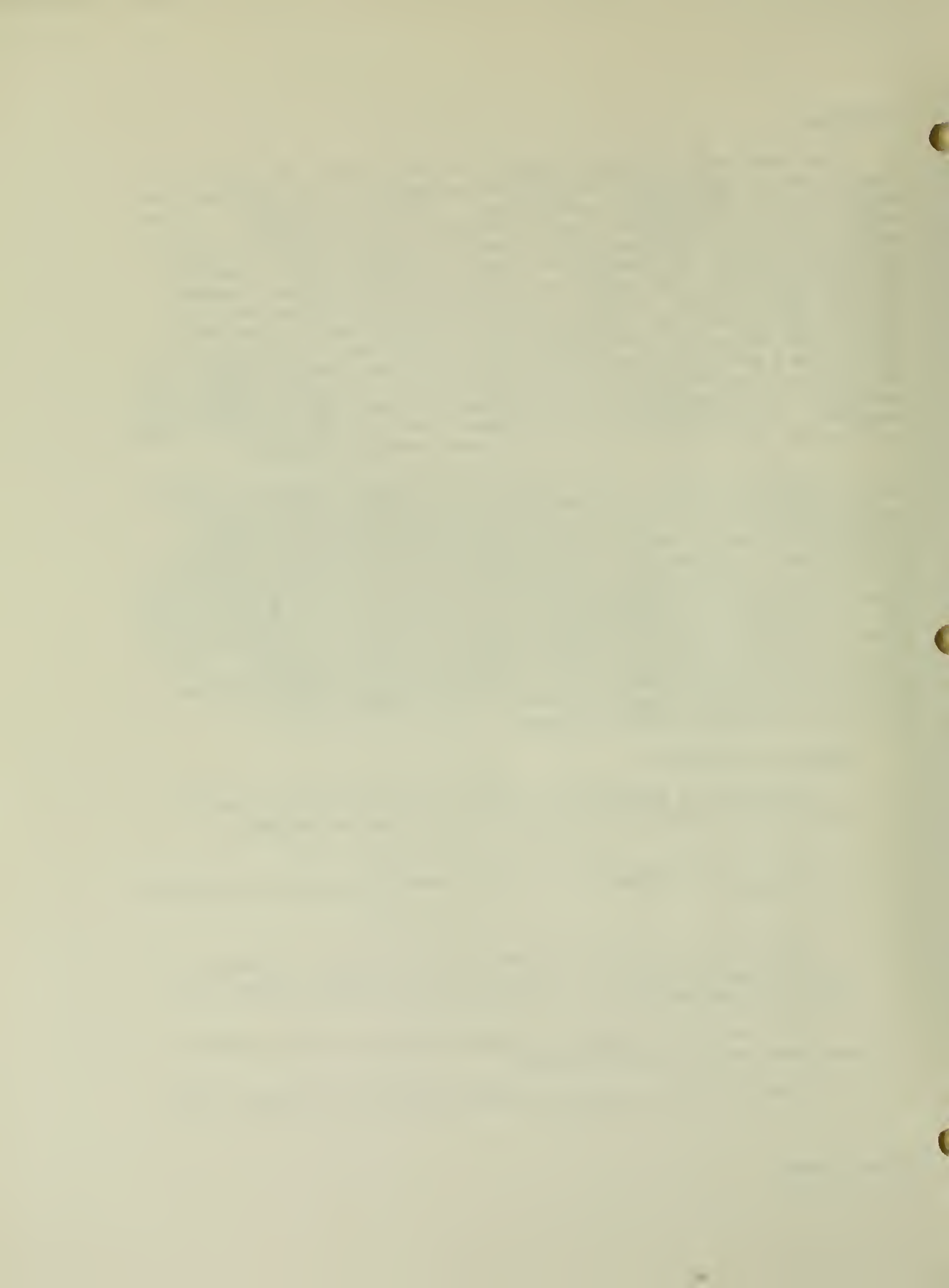
2. B and T Cell immunity in HIV infection, Brucella abortus as a probe.

3. Regulation of multiple monocyte functions, including cytokine generation by soluble and particulate stimuli and role of Fc Receptors.

4. SCID mice as a model for immunotherapy of HIV infection ***and stem cell reconstitution..***

5. Homeotic gene expression and regulation in immune cells.

FUTURE PLANS:



The Laboratory intends to continue to excel in basic research and to apply its knowledge and expertise to the regulation of monoclonal antibodies. In depth studies of immune function on the cellular level will continue. These will include specialized study of lipid metabolism, receptor-ligand & receptor-antibody interaction, adjuvant effects in promoting antibody responses (including those directed against HIV), and differentiation of immune and hematopoietic cells on the cellular and sub-cellular level. Detailed description of scientific directions are found in the project summaries for each investigator.

The staff will continue to take the lead in regulation of monoclonal antibodies and other immunomodulatory products. Products will be seen through all stages of the IND products and up to licensure and marketing. We will play a key role in shaping policy related to the complex issues of manufacture and clinical implementation of monoclonal antibodies. Education of industry on FDA policy will take place by active participation in scientific and regulatory fora and close personal contact with key representatives of industry on any important issues they face.

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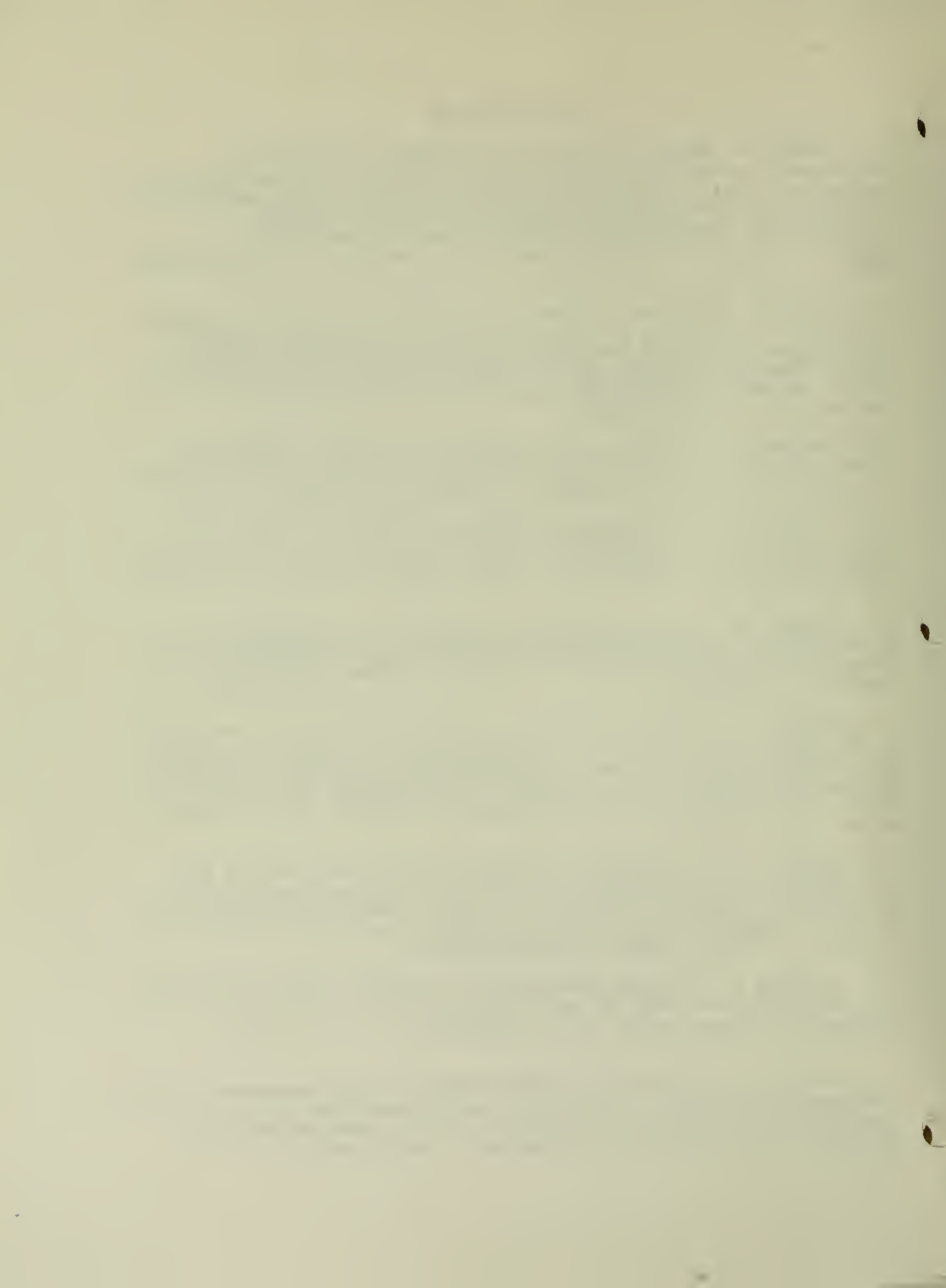

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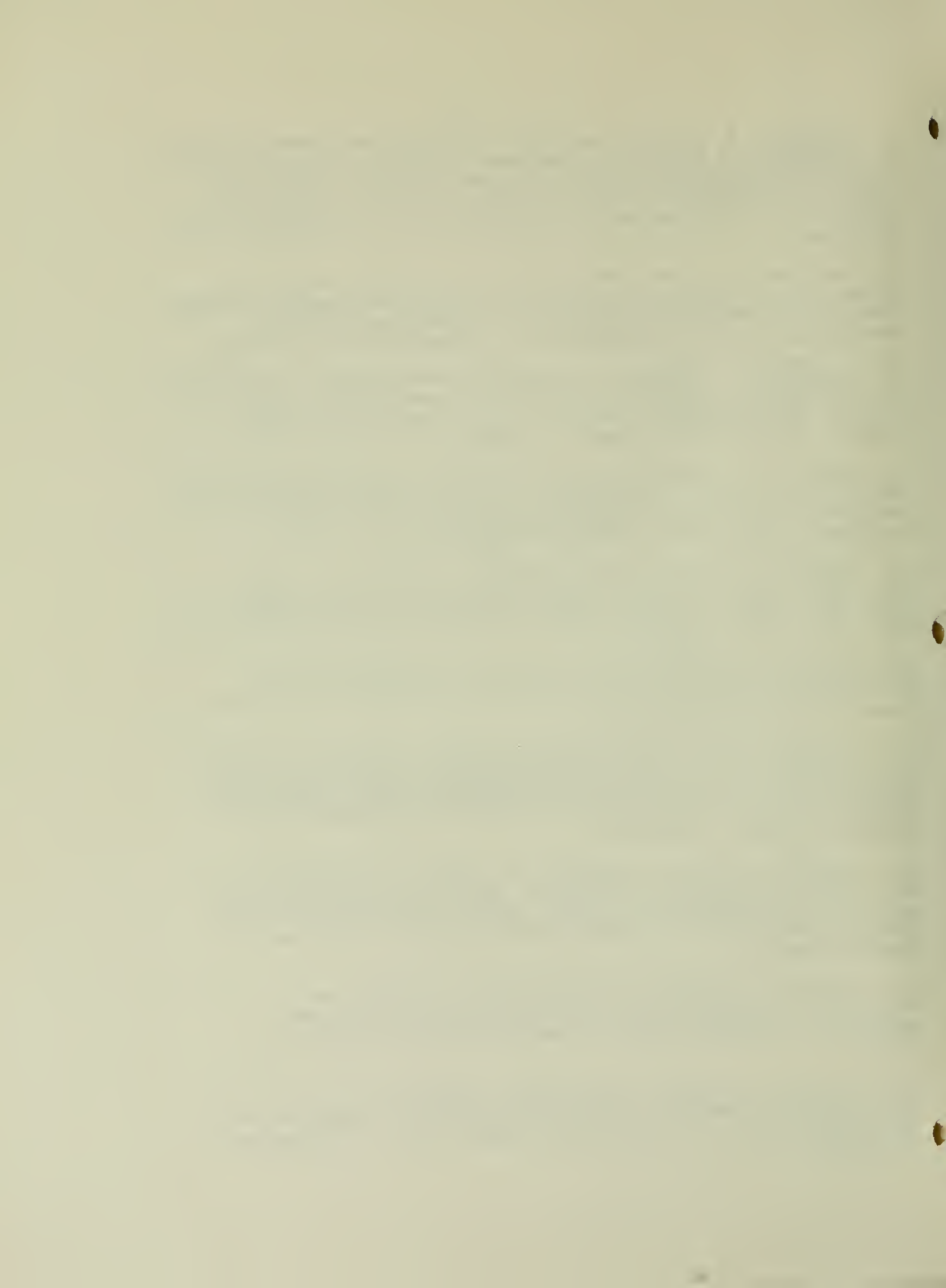

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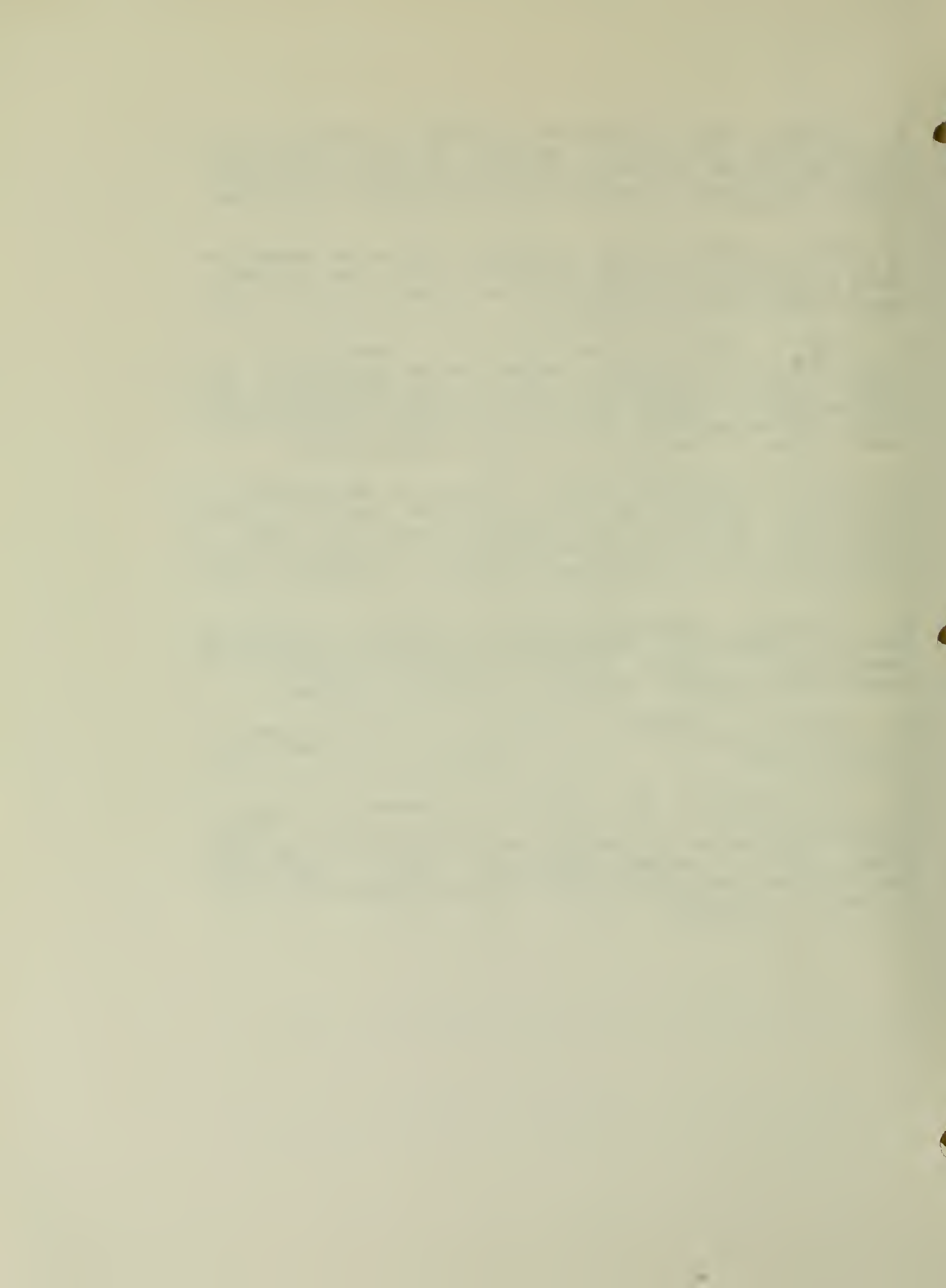

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02019-02-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Role of monocytes in adverse reactions to monoclonal antibody therapy

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

Thomas Hoffman, M.D., Chief, Laboratory of Cell Biology
Cyrstal Lee, B.A., Biologist
Ezio Bonvini, M.D., Visiting Scientist
Elaine Lizzio, B.A., Microbiologist

COOPERATING UNITS (if any)

NIH Blood Bank

LAB/BRANCH

Laboratory of Cell Biology

SECTION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	

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

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

Human monocytes released superoxide anion, prostaglandin E_2, leukotriene B_4, IL-1, and TNF when exposed t plastic surfaces coated with murine anti-CD3 monoclonal antibody, OKT3. Stimulation of mediator release by OKT3 was dependent on the amount of antibody immobilized onto wells of plastic tissue culture plates. Soluble antibody or antibody adsorbed to moncytes and reacted with an aggregating ("cross-linking") second antibody failed to induce mediator release. Monocytes "armed" with OKT3 formed rosettes with T cells in a fashion indistinguishable from that seen between monocytes and sensitized T cells. Monocytes with adsorbed OKT3 antibodies released IL-1B and TNF-a when exposed to unsensitized T cells, although increased superoxide release could not be detected. OKT4a, a murine IgG_{2a} antibody which reacts with a different T cell epitope (CD4), failed to induce cytokine the presence of IL-2 or IFN-y. These data indicate that certain antibodies bound to Fc receptors of monocytes may trigger monocyte function when reacting with cells bearing the appropriate target antigens. FcR-mediated signalling resulting in mediator release may be involved in initiating or regulating the immune response. Furthermore, systemically a administered monoclonal antibodies may induce inflammatory responses and their attendant symptomatologies via their interaction with FcR-bearing inflammatory cells.

1. Objectives:

a. To identify the participation of monocyte inflammatory mediators in adverse reactions to systemically administered monoclonal antibodies.

b. To understand the role of FcR in mediating the effects of monoclonal antibodies.

2. Methods employed:

a. Mononuclear cell isolation using ficoll-hypaque gradient separation of human peripheral blood obtained from volunteer donors through the NIH Clinical Center Blood Bank is combined with countercurrent centrifugal elutriation to obtain highly purified monocyte populations. Purity is evaluated by histochemical staining for monocyte associated enzymes such as alpha-naphthyl-acetate esterase in addition to Wright's staining.

b. Autocoid production is evaluated by immunoassay for prostaglandin E2 (PGE2) or Leukotriene B4 (LtB4). Alternatively, cells are labeled with 3H-arachidonic acid (AA*), and the radioactivity released into the supernatant analyzed by HPLC and liquid scintillation spectrophotometry using an in -line detector.

c. Superoxide assay by reduction of cytochrome C.

d. Assay of Ig coating of plastic tissue culture wells using HRPO-conjugated anti-mouse antibody.

3. Major findings:

1) OKT3 adsorbed onto tissue culture plates led to release of superoxide, cytokines, and arachidonate oxidation products.

2) OKT3 stimulation could be mediated by aggregation of adsorbed immunoglobulin on monocytes by antigen on T cells and other CD3-bearing cell lines.

3) OKT4 and OKT4a failed to mediate the effect.

4) Other monoclonal antibodies against monocyte cell surface antigens could mediate similar effects.

4. Proposed course of the project:

a. Other anti-CD3 antibodies of different subclasses, including chimeric or human antibodies will be tested for their ability to mediate FcR-based inflammatory mediator release.

b. The modulation of FcR by monoclonal antibodies will be examined for its role in the regulation of mediator release.

5. Significance:

These studies represent fundamental research on monocyte function since monocytes are important mediators of inflammation. In addition these studies provide a mechanistic basis for understanding and, ultimately, preventing adverse reactions to clinically administered monoclonal antibodies. They argue for the preferred use of antibodies with modified Fc binding domains in order to preclude these reactions.

6. Publications.

1. Hoffman T, Tripathi AK, Lee YL, Lizzio EF and Bonvini E. Stimulation of human monocytes by anti-CD3 monoclonal antibody: induction of inflammatory mediator release via immobilization of Fc receptor by adsorbed immunoglobulin and T-lymphocytes. Inflammation, (in press).

2. Hoffman T, Tripathi AK, Lee YL, Lizzio EF, Bonvini E, and Golding B. Inflammatory Mediator Release from Human Monocytes via Immobilized Fc Receptor: Potential Role in Adverse Reactions to Systemic Monoclonal Antibody Therapy. Transplantation, (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02005-03-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Monocyte differentiation: Role of tyrosine phosphorylation via CSF-1 receptor

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

Joseph Puri, Ph.D., Visiting Scientist

Thomas Hoffman, M.D., Chief, LCB

COOPERATING UNITS (if any)

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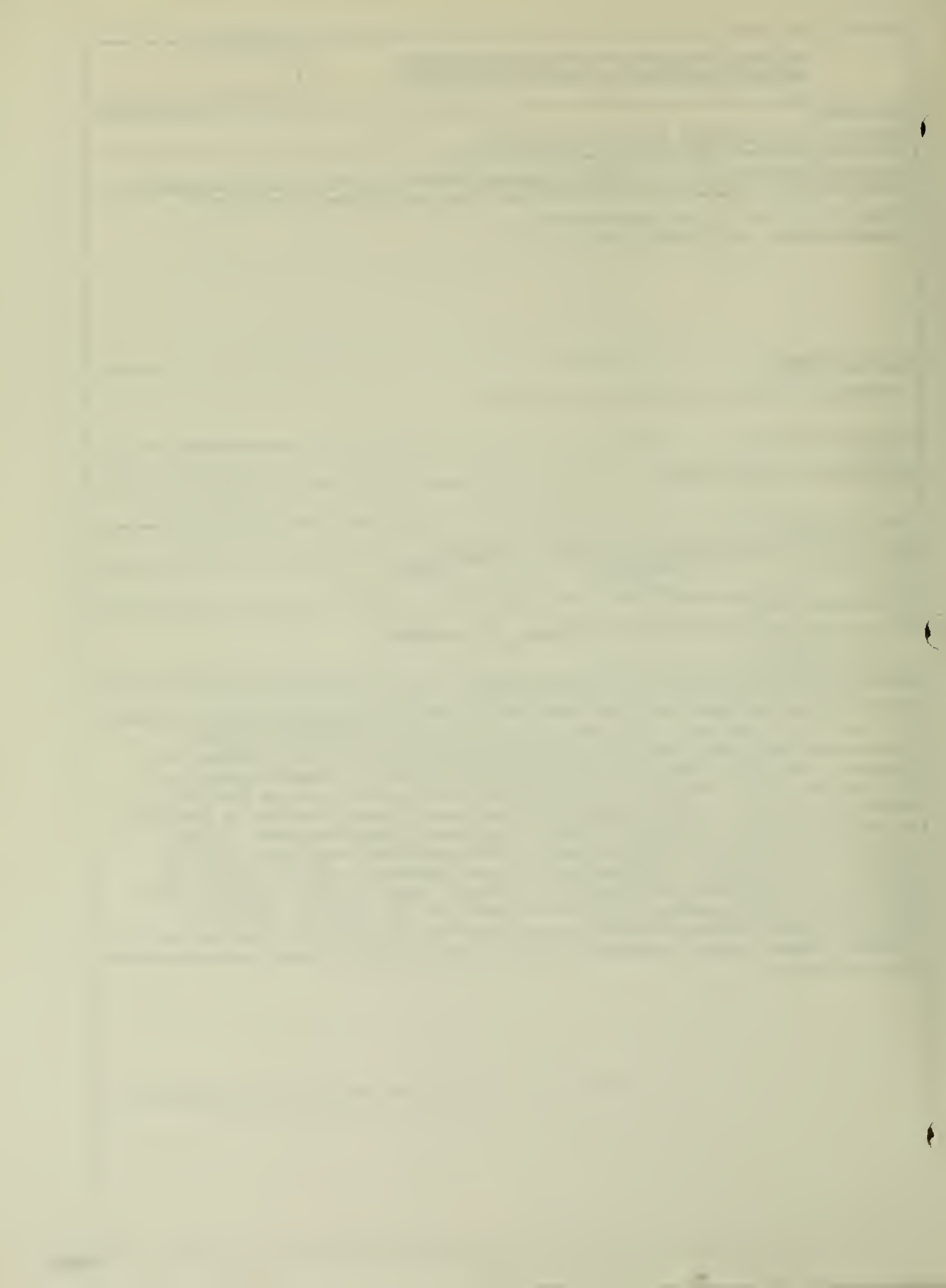
☐ (a1) Minors

☐ (a2) Interviews

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

The mouse hematopoietic cell ine, 32D, was transfected with c-fms, which enclodes for the CSF-1 receptor, a tyrosine kinase (TK). In the absence of CSF-1, transfected cells show moderate levels of arachidonic acid (A.A.) release and produce a substantial amount of prostaglandin E_2 (PGE_2) in comparison with the original cell line. Exposure of transfected cells to CSF-1, while inducing a substantial increase in arachidonate release, nevertheless resulted in inhibition of PGE_2 production. Addition of ST638, a tyrosine kinase inhibitor, to cells transfected with c-fms in the absense of CSF-1 inhibited PGE_2 within 10 to 60 minutes. Its addtion to the same cells in the presence of CSF-1 receptor may regulate cyclooxygenase activity. The different effect of CSF-1 receptor of PGE_2 production in the presence or absence of CSF-1 and the opposite effect of a tyrosine kinase inhibitor on PGE_2 suggest that both the receptor alone or the receptor-ligand complex transduce on active, but different, signal through tyrosine phosphorylation.



1. Objectives

The principal aim of this project was to investigate the role of CSF-1 receptor, a "receptor tyrosine kinase", in monocytes differentiation.

2. Rationale

Receptor tyrosine kinases (RTK's) comprise an extracellular ligand binding domain, linked by a hydrophobic membrane spanning segment to a catalytic domain capable of signal transduction involving tyrosine phosphorylation. It is believed that interaction with ligand initiates a cascade of events which may result in pleiotropic cellular responses and cell differentiation.

Although RTK's apparently play a key role in cell growth and differentiation, little is known about the precise signal transduction pathways they utilize and the mechanism of their distal action. Two characteristics common to oncogenic TK's might provide a basis for studies of normal RTK's role in cell differentiation. First, some oncogenic TK's induce a substantial increase in eicosanoid metabolism, reflected mainly in effect on prostaglandins, products of cyclooxygenase (C.O.). Second, several oncogenic RTK's phosphorylate substrates that are structurally homologous to lipocortin. Lipocortin inhibits the activity of phospholipase A_2 (PLA_2), the first enzyme in the eicosanoid metabolism pathway. Therefore, 1) transfection of CSF-1 to naive cells and 2) the production and release of A.A.and its metabolites were used in this project as a convenient and potentially relevant working model to study the role of CSF-1 and CSF-1 receptor.

Accumulating evidence indicates that external signals from growth factors regulate normal cell differentiation and maturation and that genetic alterations subverting these same signaling pathways play key roles in malignancy. The mechanism which control differentiation, commitment and maturation are less understood. One of the models to explain differentiation suggests that differentiation is at least partially controlled by external physiological stimuli.

Within the hematopoietic lineage,certain cytokines, in particular the colony-stimulating factors family (CSFs), are thought to be involved in the normal progression of hematopoietic progenitor cells to a terminally differentiated state. The exact role that these cytokines play in maturation and the sequence of events that follows the encounter between these polypeptides and their receptors has been difficult to ascertain.

Among these family of cytokines, macrophage colony-stimulating factor (CSF-1), has been implicated in differentiation. While several cytokines are known to affect a broad range of cell types within several lineages, CSF-1 activity appears to be restricted to the mononuclear phagocyte lineage. During normal hematopoiesis CSF-1 receptors

are first expressed on cells committed to the mononuclear phagocyte lineage, and the number of receptors increases dramatically as the cells continue to differentiate. The CSF-1 receptor has been shown to be identical to the c.fms proto-oncogene product. Like many growth factors receptors, it has an intracellular tyrosine kinase domain which is activated by ligand binding. This activation resulting in receptor autophosphorylation and tyrosine phosphorylation of intracellular substrates, which in turn activates different signaling and metabolic pathways. Thus, resulting in a new state of activation or in the acquisition of new cellular characteristics comprising the process of cell differentiation.

3. Methods employed

a. Cells. To study the role of CSF-1R, a tyrosine-kinase type III receptor, as it relates to A.A. metabolism, we chose the mouse interleukin-3 (IL-3) -dependent hematopoietic cell line, 32D, that had been previously transfected with either normal or point-mutated human *c-fms*/CSF-1 receptor genes. Exposure of cells transfected with *c-fms* to CSF-1 triggered proliferation and associated monocyte differentiation, which was reversible upon its removal. This system offered a special opportunity to define the role of RTK in cell activation since one could compare three different phenotypes: 1) the original line maintained in IL-3 conditioned medium (32D/il-3); 2) cells transfected with normal *c-fms* maintained in IL-3 C.M. (C.M.) (32D*c-fms*/IL-3), and 3) cells maintained in or treated with CSF-1 C.M. (32D*c-fms*/CSF-1). We tested A.A. release and eicosanoid production in unstimulated cells and also tested the effect of challenge with a calcium ionophore (ionomycin-IoM) and the tumor promoting phorbol ester, TPA, known to activate a serine-threonine protein kinase, protein kinase C (PKC). Both agents stimulate protein kinase C (PKC), enhance eicosanoid metabolism, and bypass receptor coupled pathways. Alternatively, cells were challenged with serum-treated zymosan A (STZ), a phagocytic stimulus known to activate inositol phospholipid-hydrolysis resulting in the formation of inositol phosphates and diacylglycerol (DAG), and consequential activation of PKC and Ca^{+2} mobilization. DAG may also act as a substrate that can activate PKC or be utilized to produce A.A.

b. A.A release and PGE_2 production. IL-3 dependent 32D cells or 32D transfected with *c-fms* were seeded at low density in culture medium containing, as a source of IL-3, 10% WEHI-3 (ATCC) conditioned medium (32D/IL-3 and 32D*c-fms*/IL-3) or 10% MIA-PaCa-2 human pancreatic carcinoma line conditioned medium as a source of CSF-1 (32D*c-fms*/CSF-1). Three days later, cells from each group were harvested, washed twice and transferred to 24- or 48-well plates. Arachidonic acid release was measured by incubating 10^6 cells/well of a 24 well plate in 1.0 ml Iscove's medium containing 1 mg/ml bovine serum albumin (ISC/BSA) and 2 μCi [5,6,8,9,11,12,14,16-^{3}H(n)] arachidonic acid (60-100 Ci/mmole, NEN, Inc., Boston, MA) at 37°C. Four hours later, the plate was centrifuged and two 100 μl aliquots from each experimental group were taken for estimation of uptake. The cells were

then washed twice and 0.5 ml fresh ISC/BSA medium or medium containing 20 nM 12-O-tetradecanoate phorbol-13-acetate (TPA, Sigma, St. Louis, MO) and 2 μM ionomycin (IoM, Calbiochem, San Diego, CA), or 300 μg/ml serum-treated zymosan (STZ, Sigma) was added. After an additional two hours at 37°C, two 100 μl aliquots were removed and their radioactivity determined by liquid scintillation counting in a LKB-Wallac 1218 Rackbeta counter calibrated for dpm measurement by external standard ratio.

c. PGE_2 production. 10^6 cells were incubated in 24 or 48 wells plate at 37°C with ISC/BSA alone or with stimuli. Four hours later, medium samples were removed and their PGE_2 content was assessed by radioimmunoassay of PGE_2 with a kit obtained from NEN, Inc. Assays were performed in duplicate according to the manufacturer's instructions, using standard curves constructed in ISC/BSA.

3. Major findings

We found that transfection of the normal human CSF-1 receptor into naive cells led to an activation status which appears to be opposite to that induced by the signal transduced by the same receptor coupled to its ligand. This observation was obtained in the mouse hematopoietic cell line, 32D, transfected with the normal human CSF-1 receptor encoded by *c-fms*, a receptor tyrosine kinase (RTK). Cells transfected with CSF-1R in the absence of CSF-1 show moderate levels of arachidonic acid (A.A.) release and produce a substantial amount of prostaglandin E_2 (PGE_2) in comparison with the original cell line. Exposure of transfected cells to CSF-1, while inducing a substantial increase in A.A. release, resulted in inhibition of PGE_2 production. Addition of ST638, a tyrosine kinase inhibitor, to cells transfected with *c-fms* in the absence of CSF-1 inhibited PGE_2 production within 10 to 60 minutes. Its addition to the same cells in the presence of CSF-1 induced an opposite effect, but required longer treatment (24 hours). In either cell type, A.A. release was not affected by this agent. The opposite effect of ST638 on PGE_2 production in the presence or absence of CSF-1 suggests that both the receptor and the receptor-ligand complex transduce an active, but different signal, through tyrosine phosphorylation.

4. Proposed course

Direct measurement of tyrosine phosphorylation will be assayed by western analysis to determine precisely which proteins are involved in these effects. Analogous studies will be conducted on fresh monocytes differentiating in response to a variety of agents.

5. Significance.

These studies represent fundamental research on monocyte function. They represent the basis for immunotherapy using strategies employing monocytes and cells whose

function is regulated by them. They illustrate important considerations involved in using monoclonal antibodies against cell surface components. They contribute to an understanding of the role of oncogenes in regulating malignant transformation and normal differentiation.

6. Publications.

1. Puri J, Pierce JH, and Hoffman T. Selective inhibition of PGE_2 production in cells transfected with *c-fms* encoded CSF-1 receptor genes by the tyrosine kinase inhibitor, ST638. Agents and Actions 33:314-317.

2. Puri J, Pierce JH, and Hoffman T. Transduction of a signal for arachidonic acid metabolism by untriggered CSF-1 receptor induces an opposite effect to that induced by CSF-1 receptor and its ligand: Separate regulation of phospholipase A_2 and cyclooxygenase by CSF-1 receptor/CSF-1. Prostaglandins Leukotrienes and Essential Fatty Acids. 45:43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02007-03-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

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

Joseph Puri, Ph.D., Visiting Scientist
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Maria Alava, Ph.D., Visiting Fellow
Michäel Taplits, M.D., Ph.D., Staff Fellow
Ezio Bonvini, M.D., Visiting Scientist

COOPERATING UNITS (if any)

NIH Blood Bank

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Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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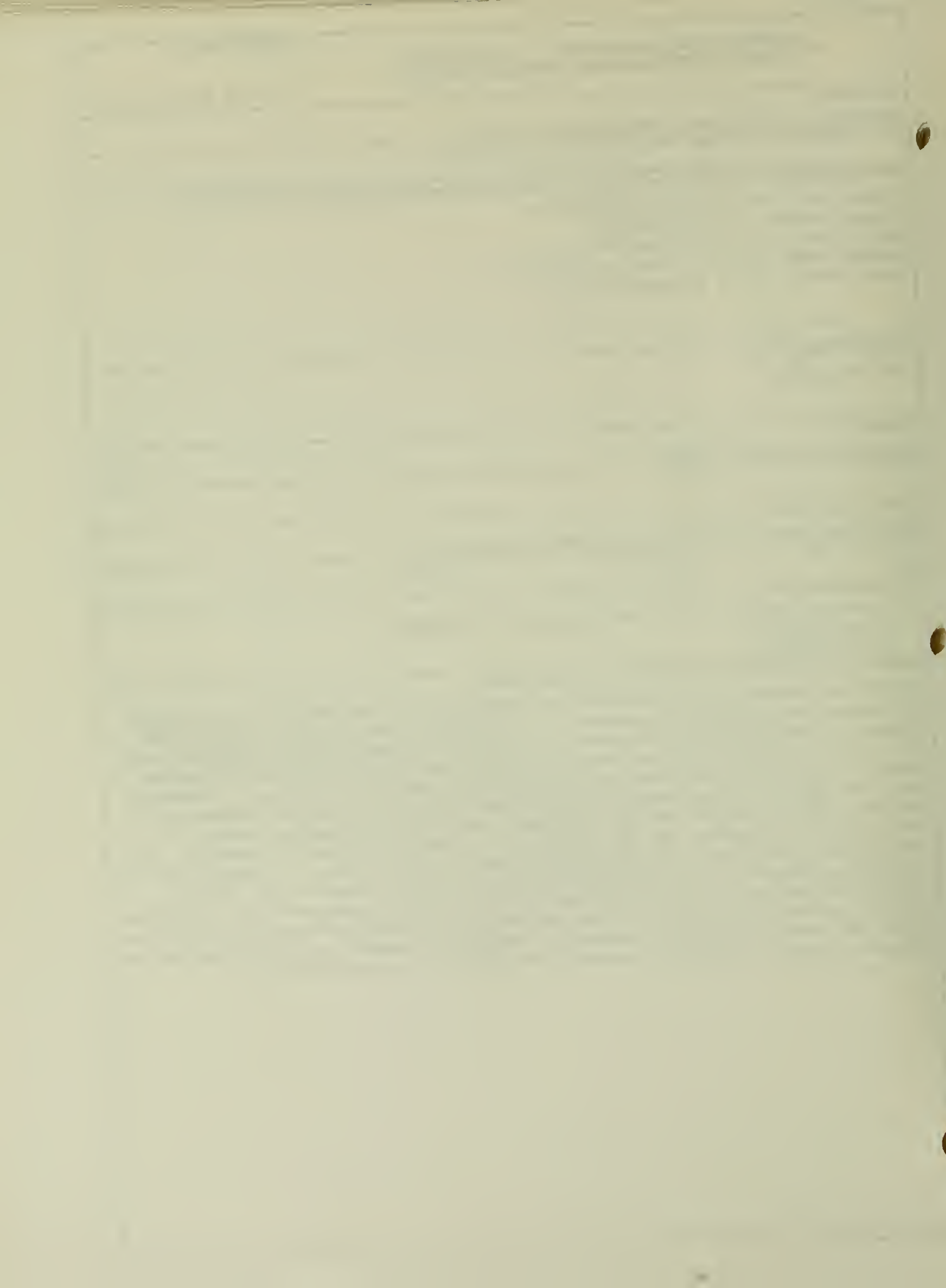
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Incubation of human elutriator-purified monocytes with anti-HLA-DR or DQ antibody inhibited the release of arachidonic acid induced by serum-treated zymosan (STZ), a phagocytic stimulus which is known to induce inositol phospholipid hydrolysis and Ca^{+2} influx. However, only anti-HLA-DR antibody partially inhibited STZ-induced inositol phosphate hydrolysis and concanavalin-A-induced Ca^{+2} influx. Incubation with anti-HLA-DR or DQ antibody inhibited phorbol ester-induced AA release as well as superoxide production and IL-1 release. Inhibition of monocyte function by anti-class II antibodies was not accompained by cAMP elevation. Furthermore, that can increase cAMP levels through different mechanisms, alone or in combination with anti-HLA antibodies, had no inhibitory effect on factor release. Our results demonstrate that perturbation of class II molecules down modulates cell activation at more than one point of the signal transduction pathway with dominant inhibition distal to inositol phosphate hydrolysis. They also suggest that the inhibition by anti-HLA Class II antibody is probably not mediated via cAMP elevation.



1. SPECIFIC AIMS

The aim of this project was to investigate the role of MHC class II molecules in signal transduction as specific activators or inhibitors of different functions of the mature macrophage i.e. synthesis activation and secretion of lymphokines, inflammatory agents and other related functions.

2. RATIONALE

Macrophage differentiation occurs as a step-wise process by which the differentiating cells acquire new functions and surface antigens that culminate in the final activated state. Membrane MHC class II molecules play an essential role in this stage. These surface molecules are involved in restricted recognition of foreign antigens in the context of self by T helper cells, and therefore are the initiators of specific immune responses. They are also involved in the proteolytic process that precedes antigen presentation and in lymphokines production and release. Taken together, these observations implies that MHC class II molecules an important candidates in the fully mature stage. It is therefore possible that these molecules may play a critical role not only in the initiation of specific immune responses, but also in the final stage of macrophage differentiation as well.

3. METHODOLOGY

The methodology and approaches which were taken to investigate the aforementioned ends were based on the use of monoclonal anti-MHC class II antibodies as the basic activating agents.

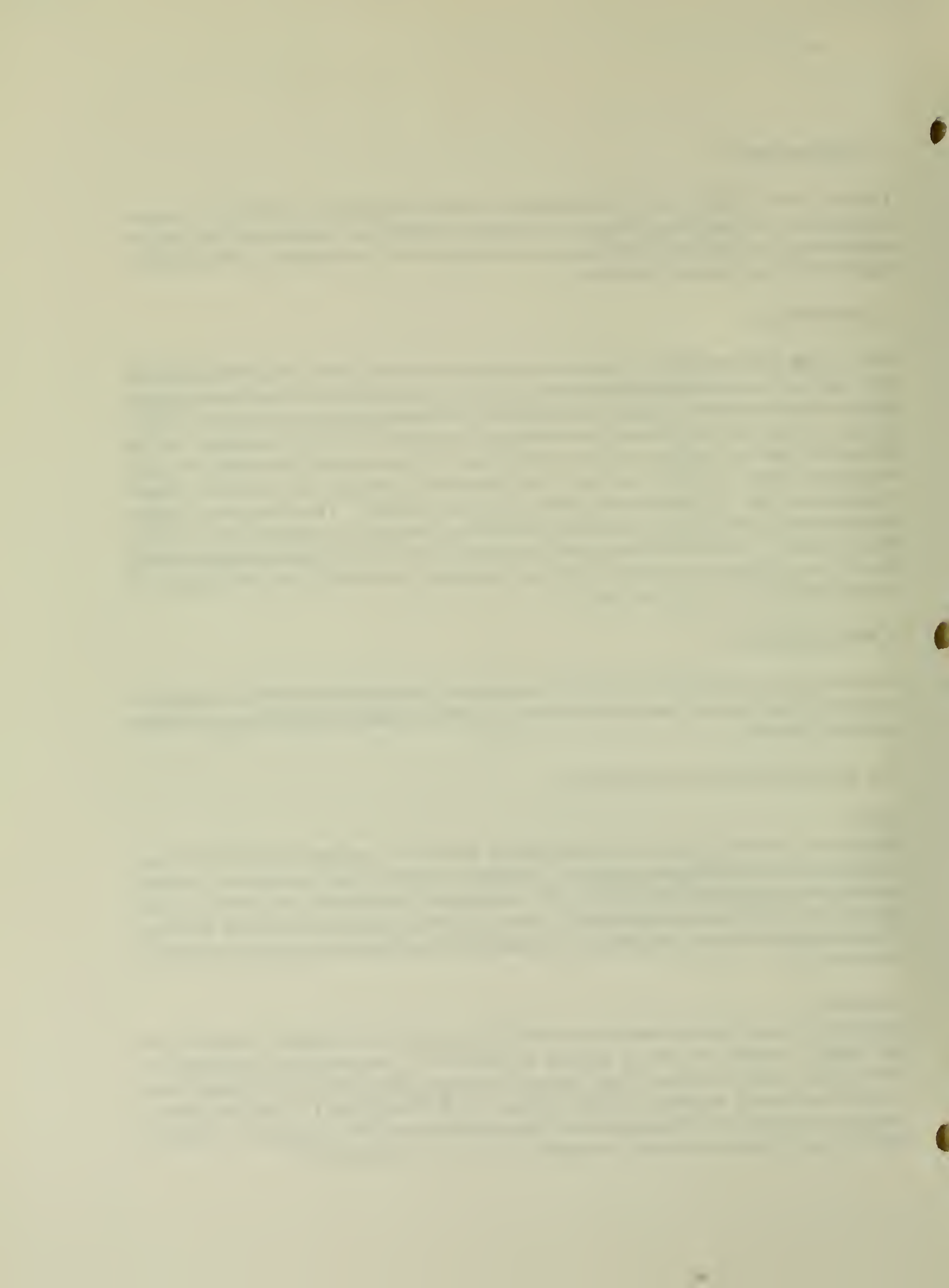
This project utilized the next methods:

Cells

Monocytes were isolated at the NIH Clinical Center by countercurrent centrifugal elutriation using techniques which are designed to yield large numbers of purified monocytes suitable for clinical use [13]. Monocytes obtained by this method were greater than 95% pure as assessed by flow cytometry and histochemical staining. Other cells consisted of lymphocytes; platelets or granulocytes were not a source of contamination.

Antibodies

Murine anti-human monoclonal anti HLA-DR (IgG_{2a}), anti HLA-DQ (IgG_1), and anti-Leu-M5 (IgG_{2b}, specific for the α subunit of the CD11c heterodimer expressed on monocytes) were purchased from Becton-Dickenson (San Jose, CA). Murine anti-human monoclonal antibody, OKM1 (IgG_{2b}), which recognizes 170 Kd cell surface antigen expressed on monocytes was purchased from Ortho Diagnostic Systems (Raritan, NJ). Human IgG was purchased from Pierce (Rockford, IL)



All antibodies were dialyzed against Isc/BSA medium with several medium exchange before use to remove preservatives.

Estimation of antibody binding to monocyte surface by FACS Monocytes were washed twice with medium prepared for flow cytometry experiments. The medium (FACS medium) consisted of PBS with 0.1% BSA and 0.02% sodium azide. One million cells were transferred to polystyrene tubes, centrifuged at 1500 rpm for 10 minutes, and resuspended in 20 μl of medium together with 20 μl of saturating concentrations of monoclonal antibodies (according to the manufacture's instructions) and 20 μl of human IgG (stock solution 10 mg/ml) at 4°C for 30 minutes. Thereafter, cells were washed twice and suspended in 1 ml medium (all steps were performed at 4°C). Fluorescence was measured by flow cytometric analysis in a Becton Dickinson FACScan.

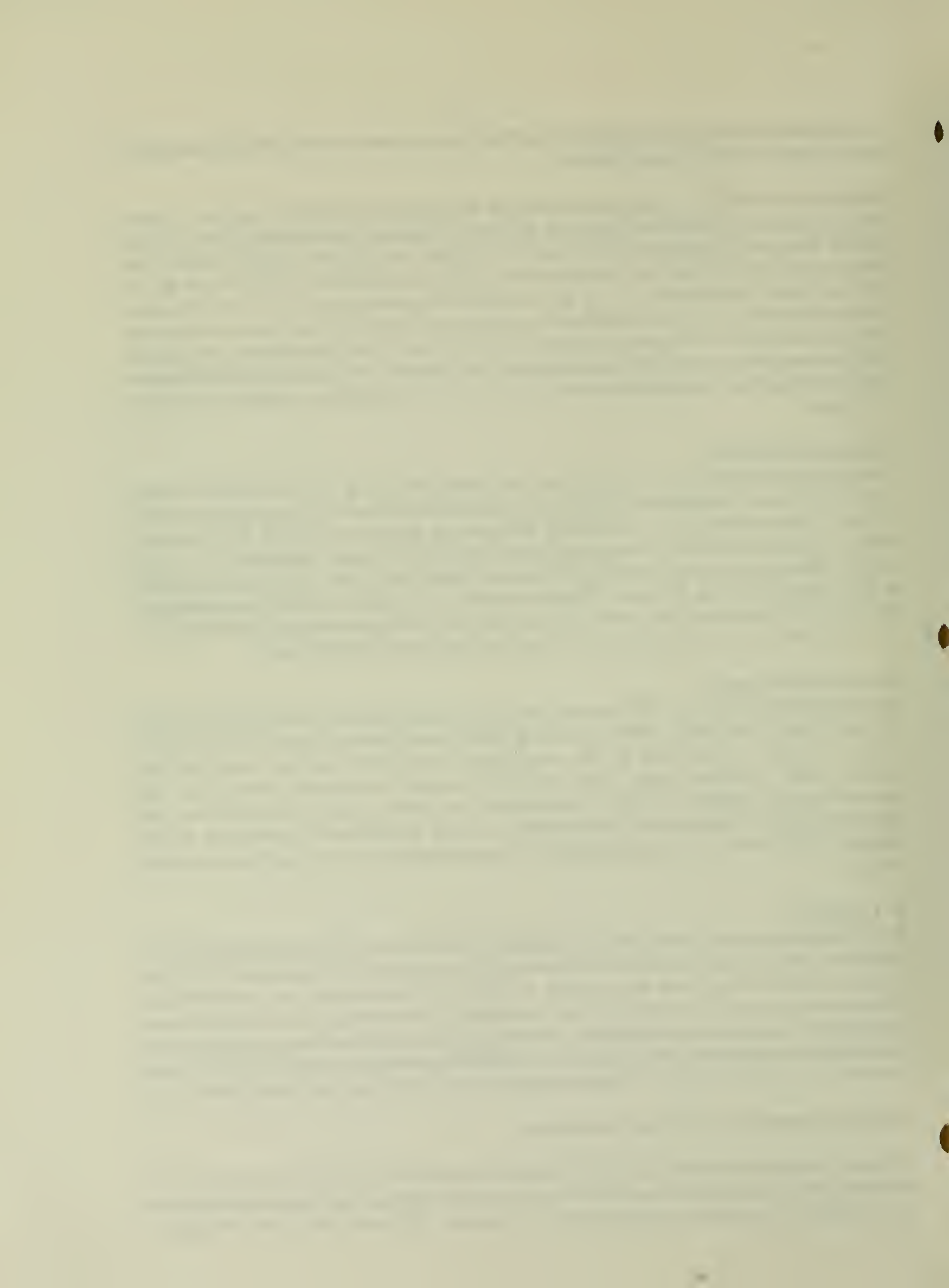
Superoxide Release
The microassay of Pick and Maizel (14) was used. Briefly, 2×10^5 elutriator-purified monocytes were incubated at 37°C with 200 μl EBSS containing 2 mg/ml cytochrome C and 1 mg/ml BSA with or without stimulating agents in 96-well flat-bottomed plates. Spectrophotometric readings at 550 nanometers were obtained in a VMax kinetic microplate reader (Molecular Devices, Menlo Park, CA) using cells incubated in SOD (300 U/ml) as a blank. Three replicates were used for each determination. Results were expressed as nmoles O_2^- per culture, using an extinction coefficient of 21×10^3 M^{-1} cm^{-1} corrected for the calculated length of the light path.

Arachidonate Release
Elutriated monocytes (2×10^6) were incubated for four hours in 24-well tissue culture plates (Costar, Cambridge, MA) containing 2 ml Iscove's medium (BSA 1 mg/ml) with 2 μCi [^{3}H]-AA. At the end of the incubation, they were washed twice and the medium was replaced with 500 μl fresh medium containing stimuli at the concentrations indicated. After an additional two hours at 37°C, the medium was removed and its radioactivity determined by liquid scintillation counting in a LKB-Wallac 1218 Rackbeta counter calibrated for dpm measurement by external standard ratio.

IL-1 Release
Elutriated monocytes (2×10^6) were incubated for 2 hours with 500 μl Isc/BSA medium containing stimuli at concentration indicated. After two hours incubation at 37°C, the medium was removed and kept frozen at -20°C until estimated by enzyme-linked immunoassay of IL-1_β kit obtained from Cistron (N.J.). Estimation of IL-1_β content was performed in duplicates according to the manufacturer's instructions, using standard curves constructed in Isc/BSA. Spectrophotometric readings at 490 nanometers were obtained in a VMax Kinetic microplate reader (Molecular Devices, Menlo Park, Ca.).

Inositol phospholipid hydrolysis estimation

Inositol phospholipid hydrolysis was measured by estimation of the accumulation ^{3}H-inositol phosphates from myo-[2-^{3}H]inositol labeled monocytes. Cells were washed with inositol free medium and pulsed for 4 hours at 37°C with 20 μCi/ml ^{3}H-myo-



inositol. Cells were washed and then incubated in the proper medium for additional 45 minutes with the indicated dose of mAb and serum treated zymosan (STZ) in the presence of Li^{+} to prevent further hydrolysis of accumulated inositol phospholipids. The incubation was terminated by the direct addition of ice-cold TCA to a final concentration of 10%.

Inositol phosphates were separated by anion exchange chromatography of the TCA soluble material using AG-1-X8 resin, 100-200 mesh (Bio-Rad Laboratories, Richmond, CA) as described previously [15]. The TCA-precipitable pellet was washed with 5% TCA, dissolved in 10% Triton X-100 and counted to estimate incorporation into the phospholipid pool. Counts were converted to dpm using an external standard ratio. Data were calculated as the percentage of the total cell-associated radioactivity of duplicate cultures.

Estimation of Ca^{+2} influx

Cells were resuspended in medium containing 2% FCS at a concentration of 10^7 per ml. Fluo-3 (Molecular probes, Eugene, Oregon) was added to final concentration of 5 μg/ml. Cells and reagent were incubated for 30 minutes with intermittent mixing. Cells were then washed twice in medium, resuspended to 5 x 10^6 per ml and were treated for 30 minutes with antibodies at 37°c. Cells were activated, just before reading, with 30 μg/ml Concanavalin A (Con-A). Intracellular calcium changes were measured by flow cytometry on a FACScan (Becton Dickenson, Mountain View, Ca) flow cytometer. Data were collected in list mode and analyzed for changes in mean fluorescence over time by CHRONYS software (Becton-Dickinson, Mountain View, CA)

cAMP assay

Monocytes (4x10^6) were equilibrated at 37°C in the presence of 300 μM IBMX in 250 μl RPMI-1640 containing 25 mM Hepes buffer, pH 7.40, 1 mg/ml BSA and 10 mM LiCl. Ten minutes later, additional 250 μl of medium, containing mAbs and/or cAMP stimulators at the concentration indicated, were added for another 10 or 30 minutes incubation. The reaction was terminated by the addition of 500 μl TCA (20%) containing 1 mM EDTA for 30 minutes on ice. Supernatant were collected after centrifugation in Eppendorf minifuge (15 minutes) and TCA was extracted by mixing with an equal volume of an water-saturated mixture of trichlorotriflouroethane (74 parts, Fisher-Scientific) and tri-n-octylamine (26 parts, Sigma) followed by a 10 minutes centrifugation at 100 x g. The upper phase from each tube was removed and concentrated in a Speed-Vac concentrator. Each sample was brought to a final volume of 200 μl with cAMP assay medium (Amersham, Arlington Heights, IL.). Estimation of cAMP levels on these samples was performed with a cAMP assay kit (Amersham) in duplicates according to the manufacture's instructions.

4. MAJOR FINDINGS

In these studies, we investigated the role of class II perturbation in elutriator purified human monocytes using monoclonal anti-HLA-DR and anti-HLA-DQ antibodies. We found that incubation with anti-HLA-DR or DQ antibody inhibited the release of arachidonic acid induced by the phagocytic stimulator serum treated zymosan, a stimulator of inositol phospholipid hydrolysis and Ca^{+2} influx. However, only anti-HLA-

DR antibody partially inhibited inositol phosphate hydrolysis and Ca^{+2} influx. Thus, we tested the effect of anti-class II antibodies at points distal to inositol phosphate hydrolysis, i.e., the induction of factor release after stimulation with TPA and ionomycin, both known to activate the second messenger protein kinase C. We found that incubation with anti-HLA-DR or DQ antibody inhibited superoxide production as well as A.A. and IL-1 release. This inhibition was not accompanied by cAMP elevation. Furthermore, addition of exogenous db-cAMP and other agents that can increase cAMP levels through different mechanism (forskolin, cholera toxin and IBMX) in combination with anti-HLA antibodies, had no inhibitory effect on factor release studied in this report. Our results demonstrate that cross linking class II molecules modulates the protein kinase C and Ca^{+2} pathway at more than one point with dominant inhibition distal to inositol phosphate hydrolysis. They also suggested that the inhibition by anti-HLA class II antibody is probably not mediated via cAMP elevation.

4. PROPOSED COURSE

The identity of the putative inhibitor of monocyte function induced by will be sought by trying to identify which proteins or other compounds are up-regulated or induced by anti-class II antibodies. the role of CD45 cross reactivity will be examined in terms of cross-linking or cross talk between these two antigen systems.

5. SIGNIFICANCE

These studies represent fundamental research on monocyte function. They represent the basis for immunotherapy using strategies employing monocytes and cells whose function is regulated by them. They illustrate important considerations involved in using monoclonal antibodies against cell surface components. They may contribute to an understanding of the role of class II antibodies in the pathogenesis of the immune defect in AIDS.

5. Publications.

1. Puri J, Taplits M, Alava M, Bonvini E, and Hoffman, T. Inhibition of arachidonic acid, superoxide, and IL-1 release from human monocytes by monoclonal anti-HLA class II antibodies: Effects at proximal and distal points of the inositol phospholipid hydrolysis pathway. Inflammation 16:31, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 BH 02003-04-LCB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism of monokine release from monocytes and modulation by antibodies

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

John J. Jessop, Ph.D., M.P.H., Senior Research Pharmacologist, DH/LCB
Thomas Hoffman, M.D., Chief, Lab of Cell Biology
Sylvia L. Henry, Lab of Cell Biology

COOPERATING UNITS *(if any)*

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Lab of Cell Biology, Division of Hematology

SECTION
Immunopharmacology Section

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TOTAL STAFF 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.)*

Two concurrent effects of serine protease inhibition by TAME (N alpha-p-tosyl-L-arginine methyl ester) on IL-1 beta release were observed in our studies by ELISA, 1) an inhibition of IL-1 beta synthesis, resulting in a decrease in the total amount of IL-1 beta available for release from the cell and 2) an increase in the percentage of total synthesized IL-1 beta that is actually released. Studies utilizing Western blot techniques to further elucidate these effects with respect the greatest inhibition occurring with the 31 kDA precursor IL-1 beta (pIL-1 beta) inside the cell. LPS alone induced production and release of the 17 kDA mature IL-1beta (mIL-1 beta), while the presence of >1 mM TAME for 6 or 18 hours resulted in release of the 33 kDA precursor IL-1 beta (pIL-1 beta) as well. This effect was also observed when TAME was added 18 hours after PLS stimulation. Since the monoclonal antibodies utilized in the ELISA recognize both IL-1 beta species, this could explain why the percentage of IL-1 beta released actually increased. The release of IL-1 beta with TAME was not accompanied by release of the cytosolic marker lactate dehydrogenase (LDH), indicating the pIL-1 beta release was unrelated to toxicty resulting in cell lysis. TNF alpha release was inhibited by 10mM TAME, and this effect was probably due to an inhibition of protein synthesis and as a result, monokine production. These results indicate that serine proteases may be involved in proteolysis of the 33 kDA pIL-1 beta and that proteolysis is probably not mechanistically involved in the signalling of mIL-1 beta release. Results further suggest that comprehensive characterization of the role of proteases in pIL-1 beta proteolysis and IL-1 beta release are required before a valid and specifc therapeutic intervention in IL-1-related disease states can be successfully developed.

1. Objectives:
   a. To utilize monoclonal antibody technology to better understand the functioning of human monocytes with respect to monokine (IL-1ß, TNFα) production and release.
      1. To determine the function of serine proteases in IL-1ß processing and release.
      2. To determine if monokine release involves the process of endocytosis.
      3. To determine the role of α2-macroglobulin in the regulation of IL-1ß production and release.
      4. To determine the role of leukophysin, a protein found in human monocytes with significant amino acid sequence homology to synaptophysin, in monokine release from human monocytes.
   b. To explore the potential use of various antibodies (anti-α2-macroglobulin, anti-leukophysin and others) to alter production and release of IL-1ß and TNFα from human monocytes.

2. Methods employed:
   a. Mononuclear cell isolation will be accomplished using ficoll-hypaque gradient separation and countercurrent centrifugal elutriation of human peripheral blood obtained from volunteer donors through the NIH Clinical Center Blood Bank.
   b. Cytokine production is measured by enzyme-linked immunoassay using monoclonal anti-cytokine antibodies.
   c. Presence of leukophysin, IL-1ß precursor and mature species, LDH and α2-macroglobulin will be determined by SDS-PAGE, Western blot and immunostaining with the appropriate monoclonal antibodies.
   d. Attempts to determine if IL-1ß is associated with leukophysin, α2-macroglobulin or LDH in the cells will be made utilizing immunoprecipitation techniques in conjunction with SDS-PAGE and Western blot, cellular immunohistochemistry techniques and light and/or electron microscopy.
   e. Attempts to associate IL-1ß with endosomes/lysosomes will be made utilizing cell fractionation techniques.

3. Major findings:
   a. LPS-stimulated IL-1ß release from human monocytes is correlated with release of a maximum of 20% of the total lactate dehydrogenase (LDH), a cytosolic enzyme with no known mechanism of release from cells with intact membranes, even though the cell membranes and function are intact with respect to trypan blue exclusion and protein synthesis

(manuscript submitted to Clinical and Experimental Immunology, May 1992).

b. The serine protease inhibitor, TAME, inhibits production of IL-1ß, does not significantly alter the release of the 17 kDa IL-1ß protein, and stimulates release of the 33 kDa IL-1ß precursor. LDH release is not increased, indicating that this effect on release of the 33 kDa precursor IL-1ß is not due to cell lysis. TAME stimulated release of the 33 kDa IL-1ß species even when added 18 hours after stimulation of the monocytes with LPS. TAME also inhibited TNF∝ release (manuscript in the F.D.A. review process July 1992).

4. Proposed course of the project:

Overall hypothesis: IL-1ß is released from the monocyte through the process of "reverse" endocytosis, in which endosomes/lysosomes (containing leukophysin) carry the IL-1ß from the cytosol to the cell surface where it is released into the surrounding milieu. This process can be regulated through the use of monoclonal antibodies.

a. We will examine the production and release of ∝2-macroglobulin by human monocytes.

b. We will study the ability of ∝2-macroglobulin, both the "slow" and the "fast" form, to regulate IL-1ß production and release.

c. We will ask if IL-1ß, ∝2-macroglobulin, leukophysin and LDH are associated inside the cell by immunoprecipitation studies, electron microscopy and/or light microscopy in conjunction with immunohistochemistry, and by cell fractionation studies.

d. We will examine the ability of the above-mentioned antibodies to alter IL-1ß and TNF∝ production and release.

5. Significance:

A number of disease states including inflammation (rheumatoid arthritis) and sepsis are mediated in part through IL-1ß and TNF∝ release from monocytes. The mechanism of production and release of these monokines remains unclear. Monoclonal antibodies provide 1) a tool to examine these processes at the cellular and biochemical level and b) a potential tool for regulation of production and release of these important monokines, which could prove to be of therapeutic significance. This project is imperative to provide a better understanding of these processes to allow for rational F.D.A. regulatory policy with respect to the monoclonal antibody industry and to help develop a rational therapeutic approach to treatment and prevention of these disease states.

6. Publications:

1. Jessop, J.J. and Taplits, M.S. Effect of high doses of morphine on Con-A induced lymphokine production in vitro. Immunopharmacology, 22:175-184, 1991. (from a project which has been terminated)

Regarding this monocyte project, one manuscript was submitted in May 1992 to Clinical and Experimental Immunology and another is going through the F.D.A. review process at present.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02004-03-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Immunological and molecular analysis on the functions of FcYRI

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

PI: Julia S. Goldstein, M.D., Fogarty Fellow, LCB, DH, FDA
Others: Jorge Laborda, Ph.D., Visiting Associate, LCB, DH, FDA
Thomas Hoffman, M.D., Lab Chief, LCB, DH, FDA

COOPERATING UNITS (if any)

LAB/BRANCH

LCB

SECTION

DH

INSTITUTE AND LOCATION

CBER, FDA, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

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

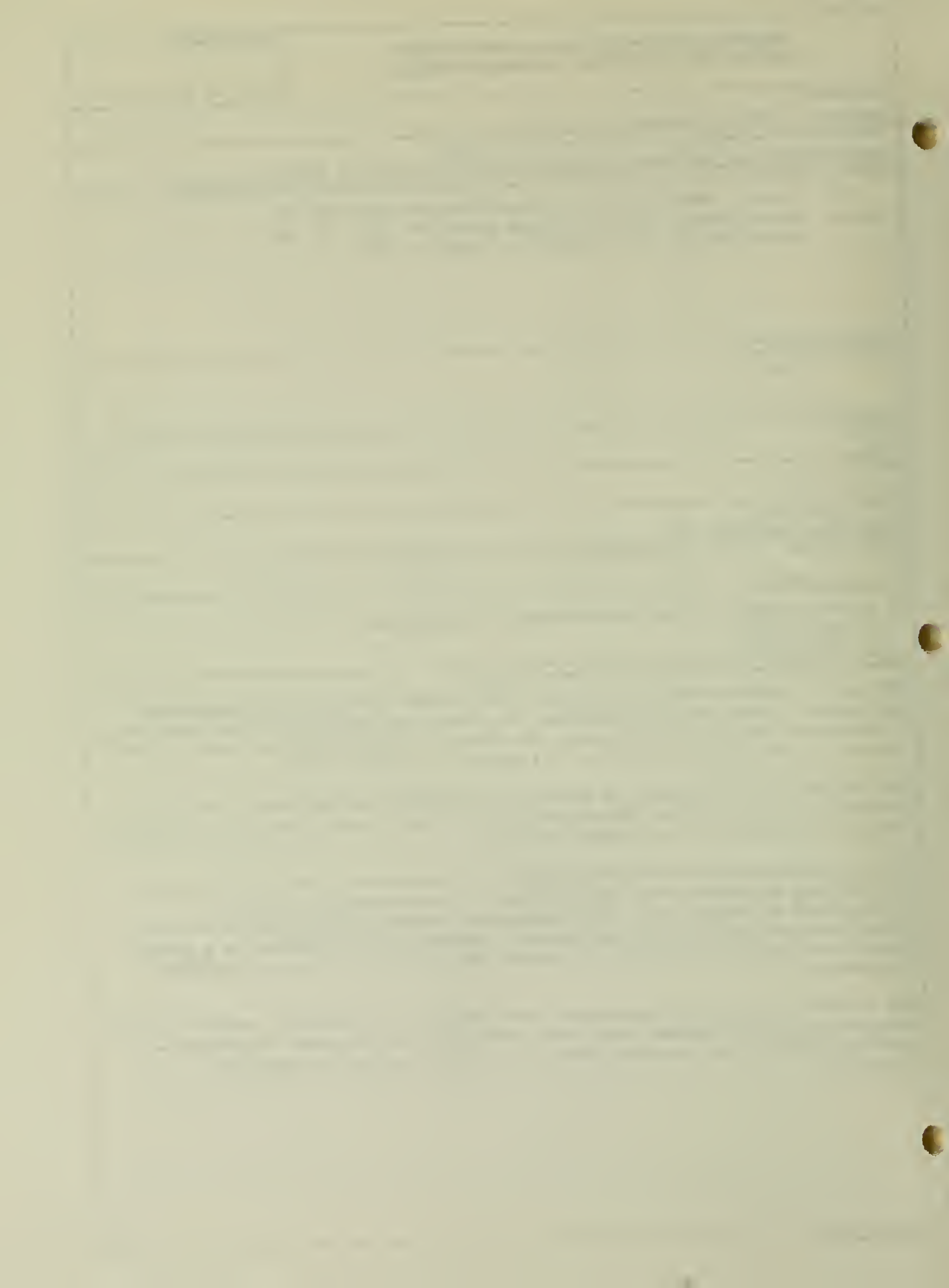
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of these studies is to analyze and examine the regulatory mechanisms implicated in ADCC (antibody-dependent cell mediated cytotoxicity) and specifically to determine the peptide sequence of the FcYRI involved in the regulation of the receptor in monocytes and cells of the monocytic lineage (U937).

The initial aim will attempt to develop a biollogical system where to study the biochemical and functional consequences of introducing modifications on the FcYRI receptor by mutation of its coding sequences.

The biological importance of this receptor is determined by its central role in monocyte and macrophage ADCC and clearance of immune complexes which is accomplished by phagocytosis and production of oxygen radicals such as nitric oxide, hydrogen peroxide and superoxide). Therefore, this receptor is a pivotal issue in the possible induction of adverse effects due to monoclonal antibody treatments.

The understanding of the determinants that regulate this receptor, specifically the interaction with antibodies, will allow the design of monoclonal antibodies with the same efficacy but decreased toxicity, so that they can be safely use clinically.



METHODS EMPLOYED:

The cell lines that will be utilized are:
U937, COS, ψ-2 and PA317 (provided through the ATCC, Rockville, Md). Human NK cells (provided by Dr.J Yodoi Institute for Virus Research, Kyoto University, Japan).

The FcγRI receptor was provided by Dr.Seed (Massachusetts General Hospital, Boston, MA).

CLONING of FcγRI into pSPD:

FcRI cloned into CMV8 provided by Dr.Seed was excised and ligated into pSPD. This vector can either be used as a plasmid or in the construction of retroviruses. The second step was to expand the clone by introducing the cDNA into transformed bacteria (DH5α E.coli from BRL). 100 ul of competent cells + 50 ng cDNA are placed 30 min on ice, then heat shocked at 42°C for 45 sec, and placed on ice for 2 min. Then 2 ml of SOC (BRL) is added and incubated at 37°C. After 1 hour, the cells are placed in agarose/ampicillin plates (NIH kitchen media), and incubated at 37°C for 24 hr. Two colonies were selected and the DNA run in minipreps to control successful transfection. The DNA was digested with restriction enzymes (Eco RI, P.C Gene Intelligenetics). Then large preparations were run with Quickgene Kit, based on ion-exchange chromatography. Then, the cDNA is ready to be introduced into COS cells by DEAE dextran transfection (Pharmacia, code # 17-0595-01)

After cloning of the receptor, the next step is the transfection of target cells. Target cells were chosen based on the following rational: a) Cos cells: were already successfully transfected by Allen and Seed and proven to rosette RBC.

b) Human NK cells: these cells are known for performing ADCC through FcRIII and the lack of FcRI. Therefor, it would be reasonably to assume that the cytolytic mechanism could be engaged with a transfected receptor.

c) U937 cells: this is a well characterized and established monocytic-like cell line. This laboratory have been working extensively with these cells in the ADCC assays and therefor are a well known model(See Major Findings).

COS cells and U937 cells (bearing endogenous FcRI) were already transfected and are in the processes of G418 selection. Two different approaches are being use:

A) Transfection of the receptor or its mutants into FcRI negative cells by either retroviral constructions (NK cells) or DEAE dextran (COS cells).

B) Inhibition of receptor expression in U937 cells using antisense oligonucleotides or knock-out by homology recombinatorial events, and successive transfection of mutants.

A) Transfection of FcγRI into U937 and NK cells by retrovirus:

The FcRI insert provided by Dr.Seed was cloned into pSPD (pSPD-FcRI) in a XhoI cloning site as previously described. The bacterial subcloning provided two colonies, sense and antisense with insert sizes of 1.4 and 1.7 Kb respectively. Previous reports showed that retroviral vectors provided a highly efficient method for gene transfer. This vector system can be divided into two components: the retroviral vector itself (which does not encode viral proteins) and the retroviral packaging cell line. During the last few years improvements in the design of both components led to gene transfer efficiencies, approaching 100% . The two type of packaging cells described,

ecotropic (ψ-2) and amphotropic (PA317) will be expose to Ping-Pong amplification. Briefly, pSPD-FcRI was transfected as a calcium phosphate precipitate into 25 cm^2 culture dish containing Ψ-2 cells. After successful transfection and passage for 7 days, ecotropic Ψ-2 cells and amphotropic PA317 cells were co-cultured in a 1:10 ratio. Because the vectors spread efficiently in the co-cultures, dominant selectable genes are unnecessary. Helper-free virions released by either cell type are cross infectious for the other cell type and the result is a back-and-forth process of vector replication and amplification that spreads through the culture. After a month of co-culturing to increase the viral titer, and when cultures were 80% confluent, supernatant were harvested. Supernatant containing viruses was added to NIH/3T3 cells in order to determined viral titer and to human cell lines (U937 cells) in order to be infected: two hundred thousand cells were incubated for 2 hr at 37 °C with 1 ml of 1:2 serial dilution of virus in the presence of 8 μg of Polybrene/ml. Cells expressing FcRI are being selected in G418 for 2 to 3 weeks and receptor expression will be measured by Facscan analysis.

COS cells transfection: Cells will be transfected with FcRI-cDNA as described previously.

. Cells expressing Fc receptors are recovered 48 hr later and expression of the receptor will be measure with FACScan using FcRI-FITC (Medarex). The mechanism by which treatment with DEAE-dextran allows mammalian cells to take up DNA and to transport it to the nuclei is poorly understood. It is assumed that large complexes containing both DNA and DEAE-dextran stick to the cell surface and are somehow taken up by endocytosis. Despite this paucity of mechanistic information, it is clear that DEAE-dextran mediated DNA transfection is a highly reproducible and efficient means to study gene expression.

B) Antisense FcRI:

U937 cells are being cultivated in RPMI 1640, 10% FBS. Cells will be cultivated with doses ranging from 40-400 μM of antisense FcRI in order to initially determine whether these doses cause toxic effects, and the optimal dose to use in further experiments. It is our estimate that it will require 3 days to be able to detect a reduction in receptor expression by Facscan and biological assays. Once this is accomplished, the cells will be transfected with FcRI mutants by retroviral vectors as described previously.

Biological activity of FcγRI-transfected cells will be studied in all cases by observing whether these cells can mediate ADCC using Cr^{51} labelled sheep red blood cells (see ADCC assay below).

Site-directed mutagenesis:

In order to define the peptide sequence of the FcγRI binding region involved in ADCC, site-directed mutagenesis by overlap extension using the polymerase chain reaction as described by Steffan N.Ho et al. and M. Smith will be used. This method represents a significant improvement over standard methods of site-directed mutagenesis because it is much faster, simpler and approaches 100% efficiency in the generation of mutant product. This protocol uses two synthetic oligos as primers to amplify a nucleotide sequence of interest. These primers anneal at either end on the target nucleotide sequence and are oriented in opposite directions. Exponential amplification of the sequence occurs over the course of multiple rounds of

denaturation, annealing and 3′ extension by DNA polymerase. Briefly, complementary oligodeoxyribonucleotide primers and the PCR are used to generate two DNA fragments having overlapping ends. These fragments are combine in a subsequent "fusion" reaction in which the overlapping ends anneal, allowing the 3′ overlap over each strand to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product is amplified further by PCR. As and additional advantage, site-directed mutagenesis by overlap extension is extremely flexible in the variety of sequence alterations that can be achieved; It allows not only the elimination of ss template and viral vector,(thus eliminating a cloning step) but also this method does not require DNA ligase. Moreover, this technique has a low rate of undesired nucleotide sequence changes.

Mutations on the receptor will be studied by FacScan analysis and ADCC assays.

ADCC assay:

Our goal is to develop a biologic system where we could study the biochemical and functional consequences of introducing modifications on the FcRI-receptor by mutation of its coding sequence. U937 cells were chosen as the primary transfectable target because they are a monocytic-like cell line, express a basal level of FcRI that increases after γ-IFN or TPA stimulation, and are capable of mediating ADCC. Thus, this model can be used to ascertain the molecular mechanisms involved in ADCC.

^{51}Chromium release assay: ADCC assays are performed by incubating 100 μl of 1640 RPMI plus 10% FCS containing 10^5 effector cells at 37°C in humidified 5% CO_2 atmosphere with 100 μl of ^{51}Cr labelled SRBC at an effector/target ratio of 1:1, together with rabbit anti-srbc antibody at a final dilution of 1/100,000. Spontaneous lysis of SRBC is determined from wells containing target cells without effectors. Non-specific cytotoxicity is determined from wells containing targets and effectors omitting antibody. Maximum lysis of target cells is measured after addition of 1% SDS. After 18 hr of incubation, supernatants were collected with a Skatron (Sterling, VA) harvester and ^{51}Cr release was determined in a LKB gamma-counter (model 1282). Specific cytotoxicity is calculated from the formula:

$$\frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}$$

Measurement of surface FcγR: One million transfected cells will be washed twice with PBS containing 0.1% BSA and 0.01% sodium azide (flow cytometry medium). The pellet is then incubated with 10 μl of anti-FcγR FITC-mAb (1 mg/ml) for 1 hr. Labeled cells are washed three times, resuspended in 1 ml of flow cytometry medium containing 1% paraformaldehyde (Sigma), and 10,000 cells from each sample are analyzed. All steps are performed at 4°C. Cytofluorimetry analysis will be performed using a Becton Dickinson (San Jose, CA) FACScan.

MAJOR FINDINGS

Preliminary studies in this laboratory showed that U937 cells stimulated with 10-50 U/ml of gamma-interferon(IFN) or 60 nM of TPA behave differently. U937 cells stimulated for 24 hr with IFN expressed higher number of receptors (as judged by FACS analysis of cells stained with the anti-FcRI antibody) as well as a slower

increase over time in cytotoxicity than cells stimulated with TPA. In contrast, TPA-stimulated cells did not show an increase in fluorescence but exhibited a noticeable increase in cytotoxicity at 24 hr.

A. Tripathi et al., established that human monocytes do not require newly synthesis of protein to mediate ADCC. Similar studies performed in our system indicate that cycloheximide (an inhibitor of protein synthesis) or actinomycin D (an inhibitor of RNA-synthesis) prevented ADCC in U937 but not in monocytes. These results indicate that newly synthesized protein is needed in U937 for ADCC, suggesting that in not fully differentiated cells the regulation of cytotoxicity is at the transcriptional and translational level. Interestingly, FACS analysis performed in parallel samples showed increase in fluorescence intensity for FcRI, thus suggesting that most FcRI receptors are already pre-formed in an intracellular compartment other than the plasma membrane. Moreover, these results demonstrate that the receptor is not the only component implicated in ADCC. Regarding fully mature monocytes, our data is consistent with cells having not only proteins but messages present as well, and therefore neither cycloheximide nor actinomycin could inhibited ADCC.

In summary: a) ADCC is an early product of the differentiation process,

b) ADCC requires not only FcRI receptor but a "machinery" that presumably consist of a protein or group of proteins suggesting that ADCC is not a passive mechanism

Therefor, if ADCC is an active event, part of the receptor should be actively involved in the transduction of the signal to the cytoplasmic cytolytic system. As previously stated, our aim is to determine the FcRI sequence involved in signal transduction.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF CBER

The development of monoclonals antibodies (MAb) and their medical uses as therapeutic and diagnostic agents is a new scientific field. Merely a decade of accumulative knowledge in this area produced significant scientific advances that led to the industrial manufacturing of clinically useful monoclonals antibodies. The initial efforts were directed to develop murine monoclonal antibodies. The high incidence of adverse effects originated in humans as a consequence of exogenous administration of murine MAb (such as the endogenous production of human anti-murine antibodies (HAMA) and anaphylactic shock) prompted the development and the examination of molecular biology techniques; The production in bacteria (E.coli) of humanized specific antibodies was proven successful and clinical trials are being drafted or in progress to test these monoclonals for diagnostic and therapeutic safety and efficacy.

The principal causes of mortality in USA are neoplastic and cardiovascular diseases, septic shock, and HIV/AIDS. Therefore, efforts were made to develop MAb specific for these diseases. For example, MAb as carrier of radioisotopes to improve imaging techniques for the early diagnostic of myocardial infarction. MAb as carrier of antineoplastic agents are also being investigated; Intensive studies are being performed in reference to MAb anti-endotoxin, anti-TNF (tumor necrosis factor) and anti-IL-1 (interleukin-1) for the treatment and prevention of endotoxic shock. Furthermore, MAb anti-HIV may be useful in the diagnosis and treatment of HIV/AIDS infection, including maternal-infant transmission.

In the field of congenital diseases, monoclonal antibodies are being study as DNA-carrier molecules. Reporting in the April issue of Human Gene Therapy, Daniel Curiel, Ed Hu, and colleagues at the University of North Carolina at Chapel Hill and the Research Institute of Molecular Pathology in Vienna,Austria, described a new vector, which in addition to its potential safety advantages can carry more DNA/RNA than traditional viral vectors. In this innovative system the Fab portion of the antiviral antibody binds the envelope of inactivated adenoviruses and the Fc' bind to a chemical linker constituted by lysine amino acid units. Lysine, combines indiscriminately and spontaneously with nucleic acids,not just DNA but also RNA. The transporter consisting of the virus and its linked DNA enters the cell via a surface receptor and is taken into the nucleus via normal cellular uptake and transport processes. Once in the nucleus, the therapeutic gene can be expressed along with native host genes. The "in vitro" successful delivery of genetic material into the defective cells led to the speculation that antibodies could be useful agents in the treatment of Cystic Fibrosis. .

It would be reasonable to conjecture that treatments with genetically engineered humanized antibodies would be less likely to induce toxic effects and further testing is required before human application.

Fc receptors (FcR's) are a pivotal issue in the possible induction of adverse effects due to MAb treatment. The binding of the antibody's Fc' fragment to the macrophage/monocyte Fc-receptor (FcγRI) has been implicated in the generation of oxygen radicals, such as superoxide and nitric oxide, and Antibody-Dependent Cell-

mediated Cytotoxicity (ADCC). These cellular mechanisms are triggered when antigen-antibody complexes bind the antibody's Fc' to the FcγRI. Although the relevance of ADCC as a part of the normal immune response against infection and tumors has been known for many years, the signal transduction pathways, mechanism of action, and genetic regulation is not understood.

One of the major responsibilities of the Laboratory of Cell Biology, Division of Hematology is the regulatory aspects that involve the development of Monoclonal Antibodies. The understanding of the biology and molecular characteristic of these cellular receptors is important as well as their interaction with the different antibody isotypes and the resulting adverse effects.

The objectives of this research proposal is to analyze and examine the regulatory mechanisms implicated in ADCC and specifically the determinant peptide sequence of the FcRI involved in the regulation of the receptor. This project will test the hypothesis that the Fc-Receptor's intracytoplasmic domain contains the peptide sequence(s) responsible for the delivery of a transducing signal into the cell nucleus. If this is true, modification in the peptide sequence should block receptor activation and consequently the induction of the genes responsible for ADCC. The verification of this hypothesis by understanding the determinants involve in ADCC and Ab-receptor interactions, will allow the design of monoclonal antibodies with the same efficacy but with decreased toxic effects so that they can be safely use clinically.

PUBLICATIONS

1) Lipopolysaccharide (LPS) from Brucella abortus is less toxic then that from E.coli, suggesting the possible use of B.abortus or LPS from B.abortus as a carrier in vaccines. Apr 1992, Infect Immun, 60(4):1385-9. J. Goldstein, T. Hoffman, E. Lizzio et al.

2)Immunogenicity of lipopolysaccharide derived from Brucella abortus: potential as a carrier in the development of vaccines for AIDS. 1991.Adv. Exp. Med. Biol. 303:227-33. J. Goldstein, D. Hernandez, C. Frasch, et al

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 022020-01-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Cloning of human dlk and antibody production against dlk protein

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

Jorge Laborda, Ph.D., Visiting Associate, Lab of Cell Biology
Crystal Lee, B.A., Biologist, Lab of Cell Biology
Thomas Hoffman, M.D., Chief, Lab of Cell Biology

COOPERATING UNITS (if any)

LAB/BRANCH

Cell Biology, Division of Hematology

SECTION

INSTITUTE AND LOCATION

FDA/CBER, Bldg 29, Room 232

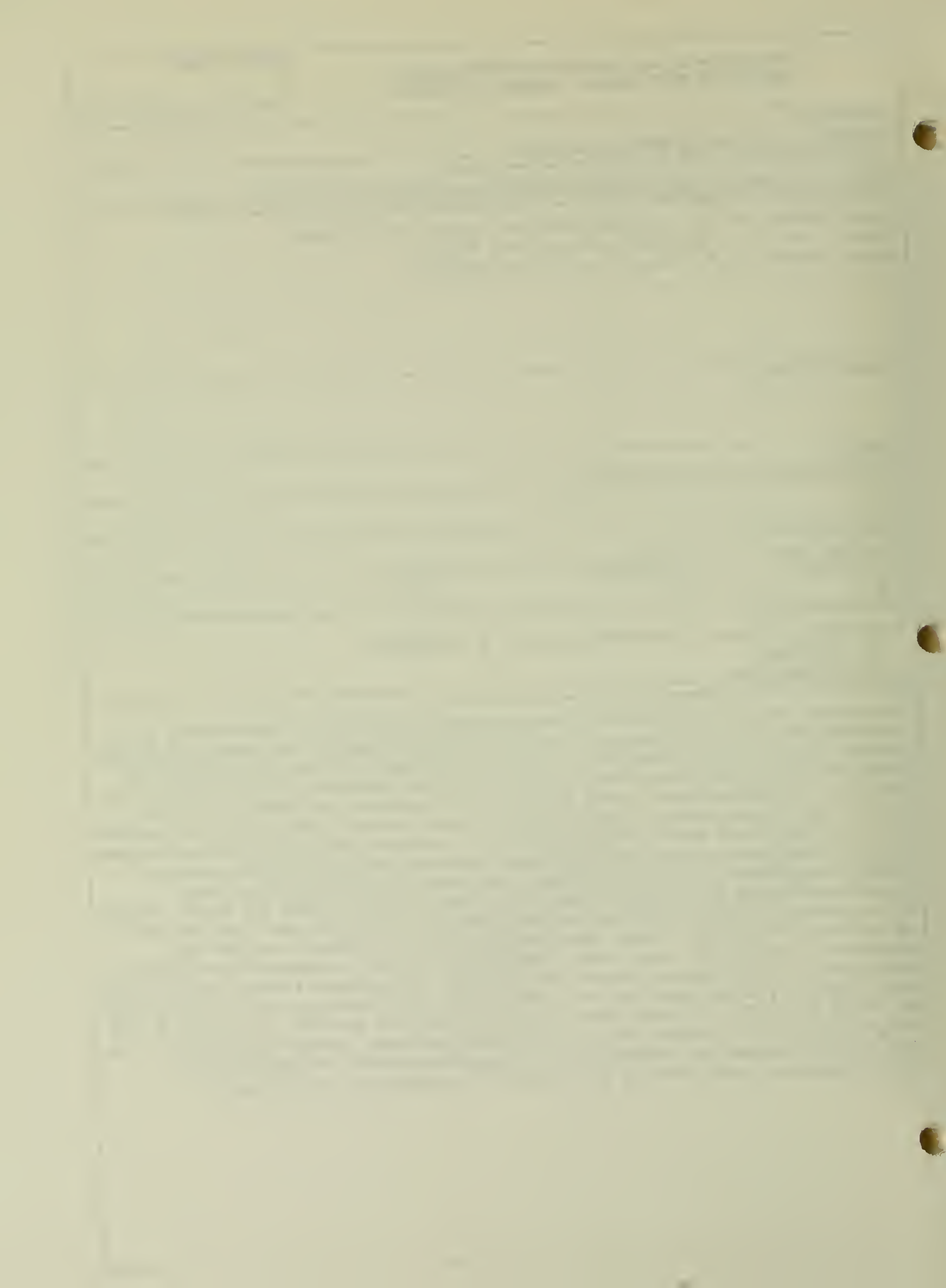
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5		

CHECK APPROPRIATE BOX(ES)

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

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

In previous work, we isolated and characterized a mouse cDNA clone encoding a new transmembrane protein belonging to the EGF-like superfamily. The predicted protein product of this cDNA (termed delta-like or dlk) contains 385 amino acids and is highly homologous to invertebrate homeotic proteins, including Delta, Serrate, and Nothch of D. melanogaster; lin-12 and glpl of C. elegans, and uEGF1 of the sea urchin. All these homeotic transmembrane proteins contain several EGF-like repeats at the extracellular domain which intervene in protein-protein interactions between cells. These interactions generate signal transduction events during normal or tumoral development which are involved in differentiation decisions. Our objectives for the year, were to obtain and characterize a human dlk clone, as well as to produce antibodies against the dlk protein. To fulfill the first objective we used two strategies. First, mRNA expression studies showed that dlk was expressed in Small Cell Lung Cancer, Neuroblastomas and, in normal tissue, almost exclusively in human adrenal gland and placenta. We screened a human adrenal gland cDNA library by using mouse dlk as a probe. Following this approach, we were able to obtain two cDNA dlk clones, that, however, were only partial. To obtain a full length human dlk, we decide to use a PCR based strategy. Computer analysis of the dlk sequence allowed the design of two oligonucleotides to be used for PCR amplification of human adrenal gland cDNA. This approach was successful.

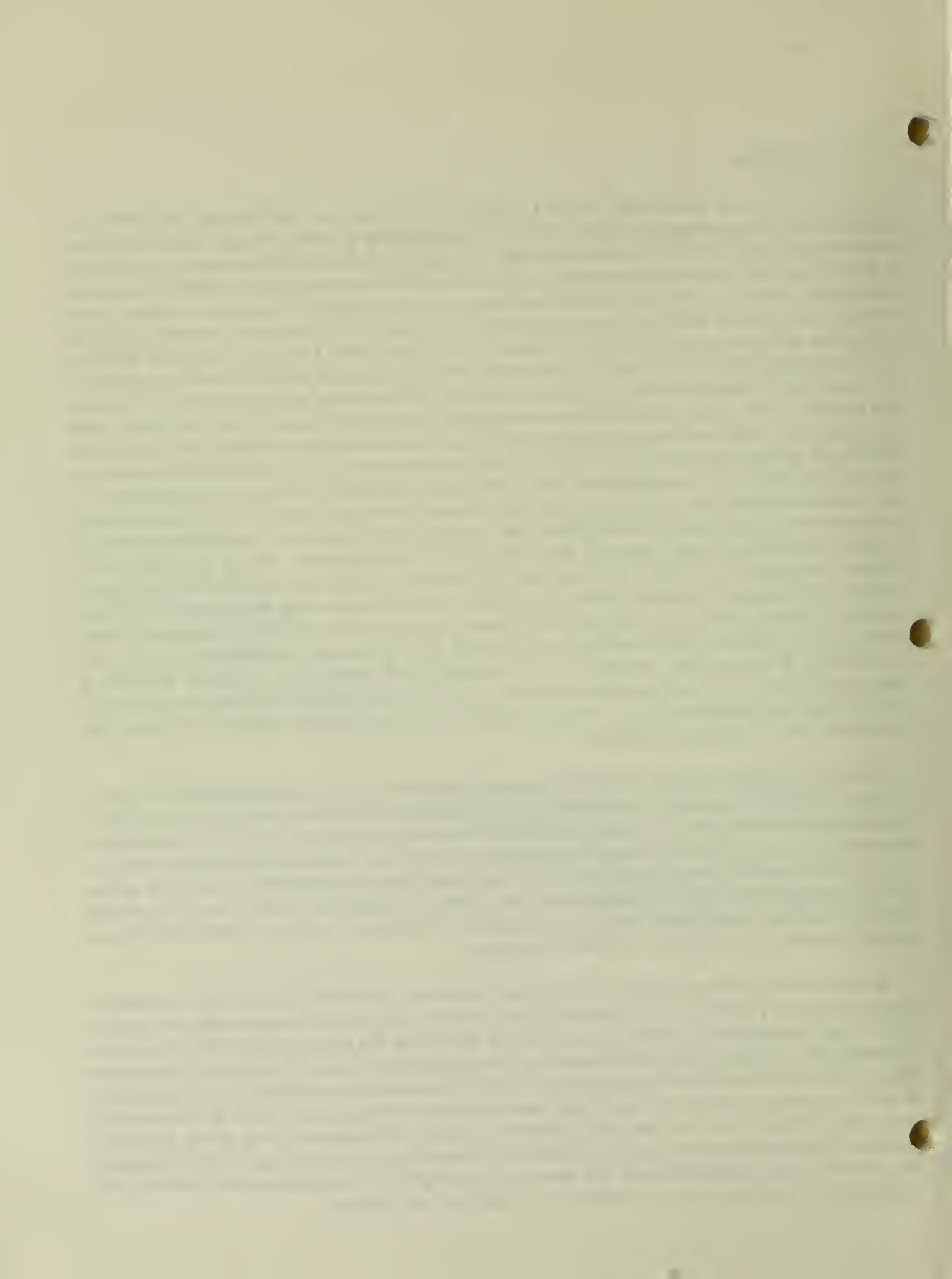


1.- Introduction

In previous work performed before I joined the laboratory of Cell Biology at CBER, we isolated and characterized a mouse cDNA clone encoding a new putative transmembrane protein belonging to the EGF-like superfamily. This cDNA was cloned because of our interest in identifying new molecules associated with the Gastrin Releasing Peptide (GRP) responsive phenotype. GRP is a mitogenic peptide that acts via a G-protein coupled receptor. The mitogenic activity of GRP has been characterized in Swiss 3T3 fibroblasts, normal epithelial lung cells, and some Small Cell Lung Cancer (SCLC) cell lines. On the other hand, BALB/c 3T3 fibroblasts and some other SCLC cell lines are unresponsive for GRP. For these reason, we searched for cDNAs which, in addition to being differentially expressed between Swiss and BALB/c 3T3 fibroblasts, were also expressed in GRP-responsive SCLC cell lines. This approach was based on the assumption that gene products related with the GRP-responsive phenotype should be missing from BALB/c and unresponsive SCLC cell lines but would be present in Swiss 3T3 fibroblasts and responsive SCLC cell lines. Following this rationale, we were able to clone a new 1.6 kbp cDNA from mouse Swiss 3T3 cells. The predicted protein product of this cDNA (termed *delta-like* or *dlk*) is highly homologous to invertebrate homeotic proteins, including *Delta*, *Serrate*, and *Notch* of *D. melanogaster*; *lin-12* and *glp1* of *C. elegans*, and *uEGF1* of the sea urchin. A high degree of homology with the *Xenopus* and human homologues of *Notch*, *Xotch* and *TAN-1*, respectively, was also found. All these homeotic genes intervene in cell to cell interactions through the EGF-like repeats. These interactions generate signal transduction events during normal development which are involved in differentiation decisions. For example, null mutations of Notch produce a misrouting of the embryonic ectodermal cells of *Drosophila*, which differentiate as nervous cells instead of epidermal cells. Other mutations in the EGF-like repeats of Notch are associated with diverse phenotypes.

Structural analysis revealed that dlk possesses features of a transmembrane protein which contains 6 EGF-like repeats in the extracellular domain, a short intracellular domain, and a signal peptide at the amino terminus. All these characteristics are also shared by the EGF-like homeotic genes. In addition, the amino acid sequence and structure of the EGF-like repeats, as well as the overall structure of *dlk*, are more related to the invertebrate homeotic genes than to other vertebrate EGF-like proteins, such as EGF-precursor, TGFα, the α, β1 and β2 chains of laminin, coagulation factors, or complement proteins. All this confirms dlk as being a new member of the EGF-like homeotic proteins.

In addition of the homologies of *dlk* already described, *dlk* cDNA shows high nucleotide sequence identity (82%) to pG2, also a 1.6 kbp cDNA expressed in neuroendocrine tumors and whose expression in normal tissues seems limited to the adrenal gland and to specific differentiation stages in the development of neuroendocrine tissue. Curiously, the protein reported to be encoded for by pG2 shows no amino acid sequence similarity with mouse *dlk*. *In vitro* translation studies showed that the molecular weight of dlk (41 Kd) is in agreement with the predicted dlk, but not pG2, protein. These data suggested that either two very related cDNAs were encoding very different proteins or that sequence disagreement between the reported pG2 sequence and dlk were at the origin of the discrepancy. Cloning and sequencing of human dlk was necessary to resolve that problem.



As mentioned above, the human counterpart of *Notch*, called *TAN-1*, homologue of dlk, has been recently cloned. As Notch, This member of the EGF-like homeotic family contains 36 EGF repeats at the extracellular domain, that are likely involved in cell-cell interactions. *TAN-1* is highly expressed in spleen, thymus, and PBLs. The level of expression in spleen and thymus seems dependent upon the development stage: spleen shows higher expression that thymus during fetal life, but the expression levels are reversed in adult life. This data indicates a possible role of *TAN-1* during the development and differentiation of the immune system, and perhaps in the differentiation events that occurs during B , T or monocyte activation and differentiation.

2.- Objectives

a.- Clone and characterize the human counterpart of dlk. This will allow the resolution of the sequence conflict between dlk and pG2. In addition, the obtention of a human clone is of great interest for the production of specific anti human dlk antibodies.

b.- To obtain anti-dlk antibodies from peptides derived from the dlk protein sequence, likely to be antigenic determinants, as defined by several physical-chemical criteria.Anti-dlk antibodies may be of importance for the diagnostic of therapy of SCLC. They also will be a valuable tool to study the possible interactions in which dlk is likely involved.

c.- Initiate the study on the possible role of *TAN-1*, a protein also belonging to the EGF-like homeotic genes, in the development and activation of the immune system.

3.- Methods employed

We used a low stringency cDNA library screening procedure by using mouse dlk cDNA as a probe. PCR methodology was also employed. Computer oriented analysis of the dlk and pG2 sequences allowed us to select two primers that, based in sequence similarities, will enable the cloning of both, pG2 and human dlk, in the case of both being different genes. Southern and Northern analysis, subcloning, plasmid preparation, and DNA sequencing of plasmid DNA were performed following standard procedures.

To produce anti-dlk antibodies, the dlk protein sequence was analyzed to search for portions of the molecule likely to be antigenic determinants. Three peptides, two in the intracellular part, and one in the extracellular part, were selected and synthesized to immunize rabbits against them. Antisera were collected and analyzed for binding antibodies by four different methods: Immunoprecipitation, immunofluorescence, ELISA and western blots.

To clone a partial cDNA from human and mouse *TAN-1* (counterparts of Notch), PCR amplification was also used. In the case of human *TAN-1*, the primers were selected through computer oriented analysis of the published sequence. Mouse *TAN-1* was cloned by using PCR primers already described in the literature.Amplified products were ligated into a plasmid, and transformed into E.coli. Plasmid minipreps were obtained and sequenced.

4.- Results

a.- Expression studies showed that dlk was expressed in SCLC and neuroblastoma cell lines, and in the normal adrenal gland and placenta. On the other hand, pG2 was also expressed in the normal adrenal gland, which was the tissue from which pG2 was cloned. For these reasons, we decided to clone human dlk from a normal human adrenal gland cDNA library constructed in the λgt10 vector. Using this approach, after two sets of screening, we were able to isolate two cDNA clones of a length of 0.9 and 0.95 kbp. As the full length dlk cDNA is around 1.6 kbp, those two clones were only partial. The results obtained with this approach seemed to indicate that the obtention of a full length clone by this method was unlikely. For that reason, we decided to approach this problem by using PCR methodology. Using this method, we were able to amplify and clone full-length dlk cDNAs from both, placenta and adrenal gland. Sequencing of the dlk clones is ongoing, but the partial sequence data obtained so far indicate that both adrenal gland and placenta cDNAs are identical and encode human dlk protein, but not pG2 protein. Some sequence discrepancies between human dlk and pG2 sequences have already been revealed. No clones encoding for the pG2 protein have been isolated. All these data seem to indicate that pG2 cDNA is the human counterpart of mouse dlk, and that the protein reported to be encoded by pG2 does not exist.
To study the species distribution of the *dlk* gene, a Southern blot analysis was performed. This analysis showed that the *dlk* gene is present in birds and mammals. However, despite the structural homology with invertebrate proteins, *dlk* gene is absent from invertebrates and low vertebrates. To our knowledge, this is the first protein homologous to invertebrate homeotic products that is exclusively present in higher animals. This suggests that *dlk* may possess a function specific to this class of animals for which the conservation of the homeotic EGF-like repeats and protein structure is important.

b.- Anti-dlk-peptide antibodies were obtained, as revealed by ELISA procedure. However, binding to the entire protein was not detected by neither of the four immunochemical methods employed. Analysis of data suggest, however, that the company which prepared the antibodies had some technical problems that may be at the origin of this behavior. Production of new antibodies is ongoing at the present and additional analysis will be needed to confirm the presence or absence of antibodies binding to the dlk protein.

c.- A 400 bp cDNA fragment of human *TAN-1* and a 450 bp fragment of mouse *TAN-1* were obtained. These fragments will be used as probes to study the regulation of *TAN-1* expression in different cell lines and normal cells of the mature and developing immune system.

5.- Proposed course of the project

The production of poly- and monoclonal antibodies is, in my view, a priority. To contend with the possibility that the new anti-peptide dlk antibodies under production are unreactive against the entire protein, strategies to express dlk protein in eukaryotic and prokaryotic systems are under consideration. The obtention of purified protein will allow the production of poly and monoclonal antibodies.Those antibodies will make possible imaging studies to radio-localize SCLC tumors in nude mice. In addition, antibodies will be valuable tools to study the possible interaction of dlk with other proteins, which will be of great importance to understand dlk function. The purification of dlk protein expressed in pro- or eukaryotic

systems will also allow to perform biochemical studies to analyze the possible enzymatic activities intrinsic to or activated by dlk.

Studies on the regulation of dlk gene expression will also be conducted. This studies will be of importance in order to understand the role of dlk in differentiation or cell growth, as well as to explore the possibility of enhancement of dlk in SCLC to facilitate radio-localization of SCLC tumor by monoclonal antibodies against dlk.

The expression of *TAN-1* in cells of the immune system, as well as the factors and cytokines affecting this expression will also be analyzed. Expression of *TAN-1* during the differentiation of cells of the immune system also will be studied.

6.- Significance

In addition to its scientific novelty, dlk is the only EGF-like homeotic gene specific to high vertebrates discovered to date, this project has important regulatory implications. First, because dlk is a membrane tumor antigen with restricted expression in normal tissues, it can be a target for monoclonal antibody-based diagnosis and therapy of SCLC and neuroblastoma.. Second, the data obtained so far indicate that dlk may be involved in the differentiation of the neuroendocrine phenotype. Particularly, dlk may be involved in tumor differentiation processes in SCLC and neuroblastoma which may be targeted by new drugs or biologicals, including antisense oligonucleotides. In consideration of the fact that the mission of FDA is protect and enhance the public health, these findings are clearly relevant and important.

7.- Publications

1.- Naval, J., Calvo, M., Laborda, J., Dubouch, P., Poiret, M., Frain, M., Sala-Trepat, J.M., Uriel, J. (1992). Synthesis of alpha-fetoprotein (AFP) and albumin and incorporation of docosahexenoic acid bound to AFP by the tissues of the fetal baboon. J. Biochem. 111(5), 649-654.

2.- Kaur, G., Viallet, J., Laborda, J., Blair, O., Gazdar, A.F., Minna, J.D., Sausville, E.A. (1992). Growth inhibition by Cholera toxin of human lung carcinoma cell lines: correlation with G_{M1} ganglioside expression. Cancer Res. 52(12), 3340-3346.

3.- Laborda, J., Sausville, E.A., Hoffman, T., Notario, V. *dlk*, a putative mammalian homeotic gene differentially expressed in Small Cell Lung Carcinoma and neuroendocrine tumors. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02006-04-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Development of A Hemopoietic Stem Cell Model

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SECTION

Division of Hematology

INSTITUTE AND LOCATION

CBER/FDA

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

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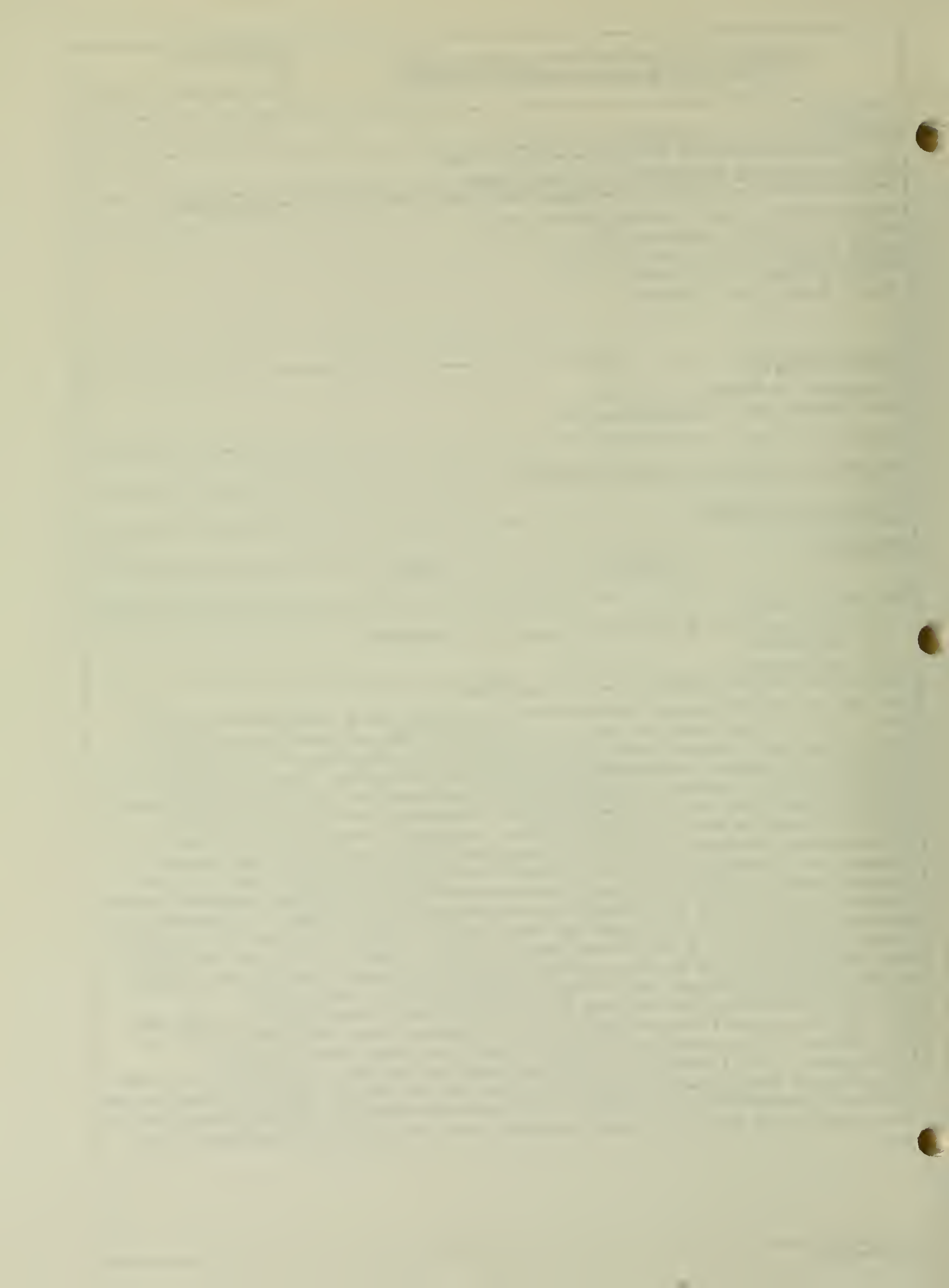
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The over all objectives of this project is to establish a hemopoietic stem cell model which can be tested to study and understand the mechanism of self renewal and/or commitment to differentiated cell types by a primitive cell. A high incidence of lineage, undifferentiated cells was identified in the spleens of female NZB mice. Phenotyic, morphological, histochemical, genomic and functional characterization of the purified population suggested that these may be very primitive cells of the hemopoietic system. In Vitro, these cells were found to differentiate in reponse to a few early-acting lymphokines and a bone marrow stromal cell conditioned medium. In that, both myeloid (adherent mac-1$^+$), and lymphoid (CD3$^+$, surface Ig$^+$), cell type were detectable. In vivo, these cells were found to differentiate and reconstitute immunodeficient SCID mice. Circulating immunoglobulines of both IgM and IgG class were detectable by ELISA in the seara of reconstituted mice 6, 8 and 12 week post transfer. Mac-1$^+$, CD3$^+$, and sIG$^+$ cells were detectable in the spleens of these reconstituted mice. During the present reporting period (October 1991 through September 1992), the in vivo reconstitutability of splenic stem cells were further investigated in comparison with the stem cells from bone marrow, mature splenic cells originating from the same donors. In parallel, similar cell types from BALB/c mice were also investigated. By 12 weeks post transfer, both bone marrow cells and splenic stem cells but not splenic mature cells) from both NZB and BALB/c mice were found to be completely reconstituted\ In that, B220$^+$ were detectable in the peritoneal cavity. The reconstituted mice were found to mount immune response when challenged with SRBC.



The over all objectives of this project is to establish a hemopoietic stem cell model which can be tested to study and understand the mechanism of self renewal and/or commitment to differentiated cell types by a primitive cell. A high incidence of lineage$^-$, undifferentiated cells was identified in the spleens of female New Zealand Black Mice. Phenotypic, morphological, histochemical, genomic and functional characterization of the purified population suggested that these may be very primitive cells of the hemopoietic system. A comparative and quantitative restriction fragment mapping analyses of the genomic DNA confirmed the germ line configuration of the genome of these cells. In Vitro, these cells were found to differentiate in response to a few early-acting lymphokines and a bone marrow stromal cell conditioned medium. In that, both myeloid (adherent mac-1$^+$), and lymphoid (CD3$^+$, surface Ig$^+$) cell types were detectable. In vivo, these cells were found to differentiate and reconstitute immunedefficient SCID mice(lack functional T and B cells). Circulating immunoglobulins of both IgM and IgG class were detectable by ELISA in the reconstituted mice 6,8 and 12 week post transfer. Mac-1$^+$, CD3$^+$, and sIg$^+$ cells were detectable in the spleens of these reconstituted mice. During the present reporting period (October, 1991 through September, 1992), the in vivo reconstitutability of splenic stem cells were further investigated in comparison with the stem cells from bone marrow, mature splenic cells originating from the same donors. In parallel, similar cell types from BALB/c mice were also investigated. By 12 weeks post transfer, both bone marrow cells and splenic stem cells but not splenic mature cells) from both NZB and BALB/c mice were found to be completely reconstituted. In that, B220$^+$ (B cells) and CD3$^+$ were detectable in the peritoneal cavity. Further, all the reconstituted mice were found to mount immune response when challenged with SRBC. In that, hemagglutinating antibodies of both IgM and IgG classes were detected in the immune sera from all the reconstituted mice, comparable to the levels from control BALB/c mice. These results conclusively establish that the splenic undifferentiated hematopoietic cells have long term repopulating potential, and are able to reconstitute the lymphoid arm of immunodeficient SCID mice. Further, to study the clonal analysis of stem cells in vivo, neonatal NOS X NZB F1 (bred in house) mice were injected with graded dose of splenic stem cells and bone marrow cells. The recipient F1 mice are being typed for all the lineages of the donor origin by two-color FMF analyses. Experiments are underway to define the in vitro culture conditions to support long term proliferation and differentiation of these cells, and to investigate the early transcriptional events occurring in these cells in response to the inducing stimuli.

Objectives:

1. To examine the long term in vivo reconstitution of immunodeficient SCID mice by splenic stem cells in comparison to bone marrow cells.

2. To study the comparative clonal analyses of splenic stem cells and bone marrow cells in neonatal NOS X NZB F1 mice and to demonstrate that both myeloid and lymphoid cell types are derived from the donor stem cells.

3. To develop in vitro culture conditions to maintain stem cells in proliferation and to study their differentiation capabilities.

4. To examine the early gene expression in the stem cells in response to inducing stimuli.

Methods:

Spleens from age matched female NZB and BALB/c mice (retired breeders) were used for isolating the stem cells. Enriched stem cell preparations were obtained by nylon wool column passage, two rounds of panning followed by magnetic bead treatment to remove differentiated splenocytes using monoclonal antibodies recognizing lineage specific receptors on the differentiated splenocytes. Bone marrow cells were subjected to magnetic bead treatment to remove differentiated cells. The purity of the resultant population was assessed by FMF analyses which was found to be 99%. These highly enriched cells were directly used for in vivo reconstitution experiments. For in vitro experiments, the enriched cells were labelled with a cocktail of flouresceinated lineage specific antibody reagents and cell sorted to obtain pure (99.9%) stem cells.

Purified stem cells were cultured in the presence of recombinant
IL-1, IL-3, IL-6 and a bone marrow stromal cell conditioned medium along with RPMI 1640 supplemented with fetal calf serum (Hyclone) for 5 days. The cells were washed in HBSS containing 0.1% BSA and 0.01% NaN3, stained with flouresceinated anti-Mac1, anti-CD3 and goat anti-mouse antibodies and analyzed by flow cytometry .

Enriched splenic stem cells, mature splenic cells and bone marrow cells (10^5) from NZB and BALB/c mice were injected i.v. into immunodeficient SCID mice. Animals were bled before and 4, 6, 8 and 12 weeks post transfer, and the sera was analyzed for circulating IgM and IgG levels by ELISA. Three animals from each group, 4 and 6 weeks post-transfer were hemi-splenectomized and the cells from the halved spleens were analyzed for mac-1^+, $CD3^+$ and Ig^+ cells by flow cytometry. Twelve weeks post transfer, half of the mice were sacrificed and the splenocytes were analyzed for their phenotype by flow cytometry and spontaneous IgM producing cells by plaque assay. Peritoneal exudate cells (PC) from reconstituted mice (12 to 38 weeks post transfer) were induced by injecting 1 ml of thioglycollate broth containing 2000 U/ml of rIFNγ and 10^{-6}M/ml of indomethacin. The peritoneal exudate cells harvested (after 5 days of induction) were incubated with uncoated magnetic beads (Dynabeads) at 37°C for 1 hour. Bead-ingested PC were removed by magnetic adherence. The non adherent cells were analyzed for the presence of $B220^+$ and $CD3^+$ cells by flow cytometry.

Mice injected with splenic stem cells, mature splenic cells and bone marrow cells were injected i.p. with 1% suspension of sterile Sheep Red Blood Cells (SRBC) and 7 days later, serum antibody to SRBC was tested by hemagglutination assay.

Neonatal NOS X NZB F1 mice were injected i.p. with varying doses (10^5 through 10 cells) of purified splenic stem cells ~~and bone marrow cells within 24 hours of their birth.~~

Message Amplification Phenotyping (MAPping) technique was employed to determine the transcriptional status of the stem cells prior to and after stimulation compared with the cell types of known lineage. This method comprises reverse transcription of total cellular RNA to derive cDNA, and amplification of specific DNA fragments by PCR. Total cellular RNA from freshly isolated stem cells was

employed to synthesize the first strand cDNA, using oligo dT as primers and AMV reverse transcriptase. Initially, primers for β-actin and IL-3 (obteined from Clontech,Inc., CA.,) were used. Typically, 100 ul of PCR mixture containing PCR reaction buffer, dNTP mix, first strand cDNA, Teq DNA polymerase and the primers were subjected to 35 cycles. The temperatures for PCR were: 95°C for denaturing for 1 min, 56°c for primer annealing for 2 min, and 68°C for primer extension for 3 min. A ramp time of 1 min was used between these temperatures. PCR amplified DNA were analyzed by Southern blot and liquid hybridization with ^{32}p labelled oligonucleotide probes. In other experiments, purified splenic stem cells were aliquoted 1, 10, 100 and 1000 cells per tube and were lysed with buffer containing 5% NP40 and first strand synthesis components including a universal Oligo dT primer and reverse transcriptase (BRL-SUPERSCRIPT kit). Aliquots of first strand synthesis mix were directly employed to amplify with specific primers such as β-actin and GAPDH, by standard PCR. Initially, the reactions were monitored at each step by radio-labelling with γ-p^{32} labelled dATP and examining the products by gel electrophoresis and autoradiography.

Results:

The flow cytometric analysis of stem cells cultured in the presence of combination of recombinant IL-1 + IL-3 + IL-6 or bone marrow stromal cell (TC-1) conditioned medium showed the development of mac-1$^+$ (33%), CD3$^+$ (14.5%) and Ig$^+$ (23%) cells.

The immunedefficient SCID mice reconstituted with splenic stem cells and bone marrow cells developed serum IgM and IgG, 6, 8 and 12 weeks post-transfer. Initially, animals receiving mature splenic cells demonstrated serum immunoglobulins which gradually decreased and disappeared by 12 weeks post transfer.

Splenic B cells producing spontaneous IgM were detectable at similar levels in SCIDs receiving either NZB or BALB/c splenic stem cells, or bone marrow cells of NZB or BALB/c. Spleen cells and PC from reconstituted mice with mice contained Ig$^+$, B220$^+$, CD3$^+$ cells by flow cytometry . No such lymphoid cells were detectable in mice injected with mature cells or saline (control).

Mice reconstituted (12 to 38 weeks post transfer) with splenic stem cells and bone marrow cells challenged with 1% Sheep Red Blood Cells (SRBC) developed antibodies to SRBC with a titer of 1:16 equivalent to that observed with immunized control BALB/c mice. Subsequent challenge, boosted the antibody titer to as high as 1:64. Both IgM and IgG class antibodies were detectable in the agglutination reaction. No demonstrable antibody titers were detected in the sera from the mice injected with mature spleen cells or saline.

These results clearly demonstrate that splenic stem cells similar to bone marrow cells, are able to completely reconstitute the lymphoid arm of the immunodeficient SCID mice. Such a reconstitution is not only of long term but also provide immune competence to the recipients.

Neonatal NOS X NZW F1 injected with varying number of stem cells are currently being typed for donor origin cells of both myeloid and lymphoid cell type.

Before stimulating the stem cells, it is essential to standardize the conditions and parameters for PCR. Initially, total cellular RNA from freshly isolated stem cells was used to make cDNA which was amplified by PCR for messages for β- actin and IL-3 messages. Electrophoretic analyses of PCR amplified DNA on composite agarose gel revealed the presence of β-actin (a house keeping gene) message, but message for IL-3 was not detectable. As the freshly isolated stem cell population will be oligoclonal in nature, techniques to detect messages at single cell level is very crucial. With the techniques described above, messages for both β-actin and GAPDH (both house keeping genes) could be detectable at single cell level.

Significance:

The findings obtained conclusively establish that splenic undifferentiated cells are true non-committed multipotential hemopoietic stem cells. Availability of large quantities of these cells from the spleens of NZB mice (20-24 x 10^6 cells/spleen) serves as a valuable source for further studies on the biology of stem cells. Using this stem cell model one can now study and determine what factors or conditions are necessary for the growth and differentiation of these cells in culture. Further, one can dissect and analyze what signalling pathways are triggered by external stimuli (lymphokines) and the resultant molecular gymnastics that lead to either self renewal or commitment to particular irreversible lineage. The use of MAPping by PCR (standardized here) enables to detect low levels of messages in a single or very few stem cells resulting from stimulation by lymphokines. An understanding of these cellular and molecular mechanisms, provides strategies to selectively manipulate the differentiation of an uncommitted hemopoietic cell to a particular lineage of interest. Additionally, the PCR technology can be used to generate recombinant and/or chimeric monoclonal antibodies for therapeutic and diagnostic purposes. The PCR technology can be further employed to detect the contaminant adventitious agents in the large scale production of antibodies. This methodology would also be highly useful in understanding the molecular events that result when monoclonal antibodies react with their specific receptors on the cell and in turn which regulatory molecules affect such differentiation.

Proposed Course:

Using MAPping technique, messages in the stem cells stimulated with lymphokines for various time points will be examined, to determine the sequence of gene activation. The primers and probes necessary for detecting the messages for TdT transferase, cyclin I, II and III, c-myc, c-fos and IL-1, and IL-3 will be synthesized in house, based on the published nucleotide sequences obtained from EMBL data base.

The role of basal membrane components such as collagen, fibrinogen and others in cohort with single or combination of recombinant lymphokines including stem cell factor will be investigated to define optimum culture conditions that support long term maintenance or differentiation of stem cells. Using this system clonal analysis

of stem cells will be investigated to dissect the long-term survival or self renewal and the limited differentiation leading to committed progeny.

In the above SCID model, only lymphoid development of the stem cells could be traced, and the development of myeloid arm is not possible to detect, in the absence of specific cell surface markers to discriminate the donor vs host population. Further, it is essential to clonally analyze the reconstitution potential of splenic stem cells in comparison with the bone marrow cells. To examine these issues, neonatal NOS X NZB F1 mice were employed as recipients. NOS mice being a low murine virus containing strain and genetically different (H-2q/s) have low NK cells. Availability to detect myeloid progenitors of NOS type while analyzing the repopulated cells.

Further, procedures to identify and isolate stem cells from human PBL's and/or cord blood will be attempted to develop human stem cell model. The biology of human stem cells can be examined in parallel with the murine model.

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Manohar, V, Brown, EM, Chused TM. Murine splenic null cell compartment contains distinct haematopoietic subpopulations: enlargement of a myeloid and an undifferentiated subset with the development of splenomegaly in New Zealand Black mice, Immunology, 1992; 75:448-55.

Manohar, V, Hoffman, T. Monoclonal and engineered antibodies for human parenteral clinical use, 1992, TIBITECH, In press.

Published proceedings

Manohar, V, Huppi, K, Hoffman, T. Splenic stem cells of New Zealand Black mice: Isolation, phenotypic,histochemical and functional characterization, 1992, Exp.Hematol, 20: 121-142. Proceedings of the symposium on stromal regulation of hematopoiesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 022021-01-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Development of Xenogeneic antibodies to murine splenic lymphocytes

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

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INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2	1

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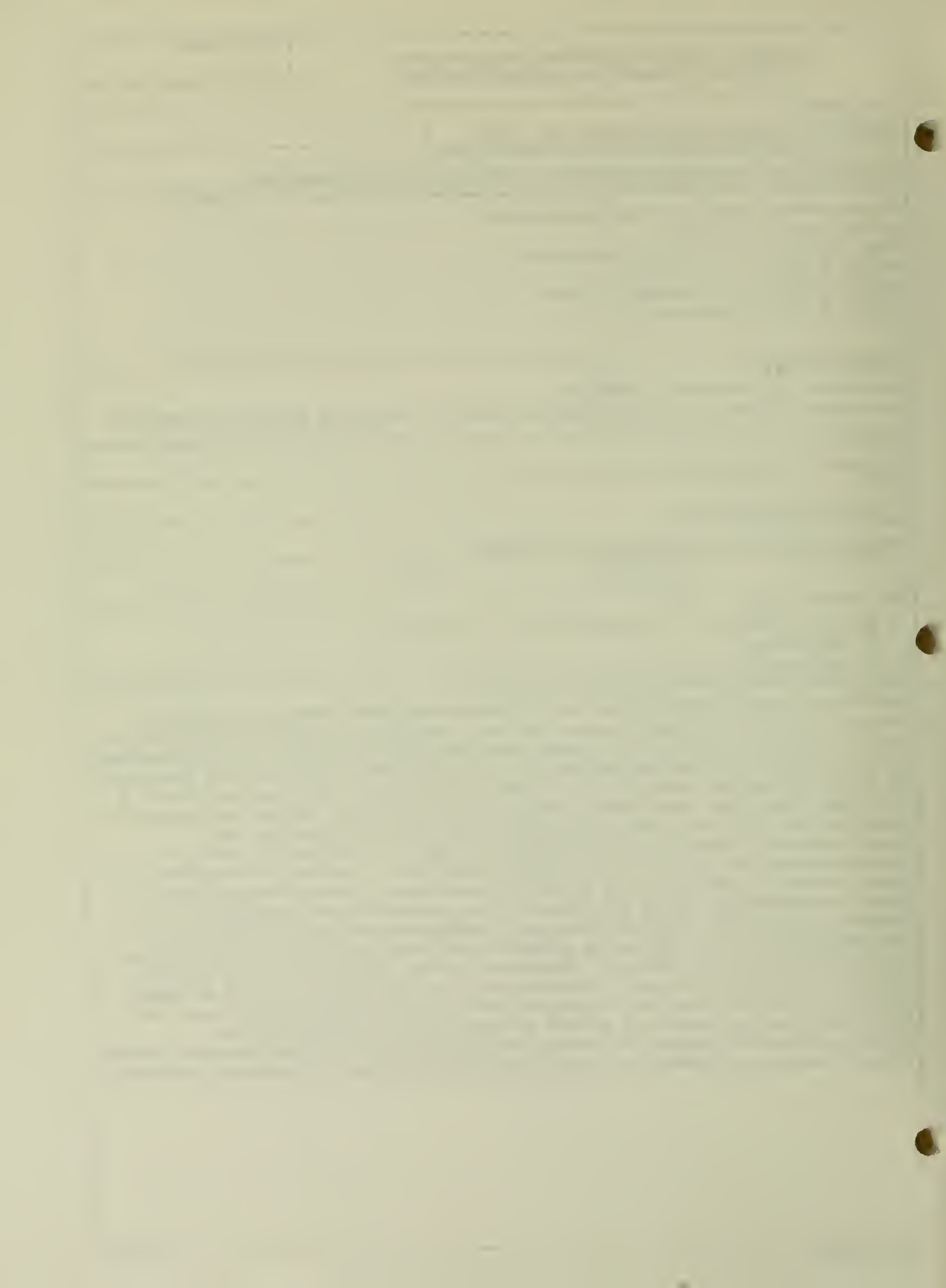
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The overall goal of this project is to develop xenogeneic monoclonal antibody reagents recognizing unique surface antigenic receptors on murine lymphocytes and their procursors. Female Wistar rates were immunized with single cell preparations of whole spleen from New Zealand Black Mice. Hybridoma cell lines were generated by fusing immune rat spleen cells with non secreting NS-1, myeloma cell line. A hybridoma clone VMM-2 was found to secrete an IgG_{2b} antibody recognizing antigenic receptors on cells from bone marrow, spleen but not from thymus by flow microflurometry. VMM-2 binds specifically to IgM^+ B cells, but the antigen recognized does not relate to any lineage specificity. Further, the antibody was found to specific for the cells of the $H\text{-}2^d$ haplotype. Genetic mapping using a panel of recombinant inbred and congenic mice, revealed that the antigenic site is located at the K end of the MHC complex. Further studies, using a series of transfected cell lines expressing chimeric $H\text{-}2K^d$ gene products on their membranes has confirmed that the antigenic determinant is the region between the residues D152 to S184, located at the C terminal end of the a2 domain of the K^d molecule. A comparative Western analyses of lysates from T, B and monocyte cells lines ($H\text{-}2^d$), VMM-2 specifically binds to a protein of approximately 180-200,000 m.w. Experiments are in progress to further determine whether the high molecular weight protein recognized by VMM-2 is really the tetrameric form of the class I molecule.



Objectives: To develop xenogeneic monoclonal antibody reagents recognizing unique surface antigenic receptors on the splenic lymphocytes and their precursors of New Zealand Black mice.

Methods:

Female Lewis rats were injected i.p. with 10^7 NZB spleen cells mice three times at weekly intervals. Three after the third injection, the rat spleen cells were fused with exponentially growing 8-azaguanine-treated, non-secreting PANS-1 myeloma cells in the presence of 35% polyethylene glycol. The fusion mixture at a cell density of 2 X 10^5 cells/ well were initially cultured in the HAT medium, to select hybrid colonies. The supernatants from the wells containing hybrid cells were screened for the antibodies binding to NZB spleen cells, by flow microfluorometry (FMF). Hybrid cells from positive wells were cloned by limiting dilution on irradiated BALB/c thymocytes as feeder layer.

Culture supernatants from positive clones were purified by DEAE column chromatography followed by gel filtration. Purified antibody was typed to be of a rat $IgG2_b$ antibody by using a monoclonal isotype typing kit and conjugated with fluorescein isothiocyanate by standard methods .

The tissue distribution of the antigen recognized by VMM-2 was determined by testing the cells from marrow, lymph nodes, spleen and thymus. The relationship of VMM-2 determinant to other cell surface antigens was tested by a series of blocking experiments, wherein, the percentage blocking of the antigenic site by various antibodies was calculated. To determine whether the putative antigen is an activation antigen, spleen cells from BALB/c mice stimulated with LPS were employed. To determine whether glycosylation of the antigenic determinant would affect the nature of VMM-2 binding, the target cells were either endogenously treated with tunicamycin or in vitro treated with endoglycosidase . To determine whether, VMM-2 is recognizing a circulating or cytoplasmic antigen, either target cells were either blocked by NZB serum and stained by VMM-2 or the cells fixed by methanol were stained by FITC labelled FITC.

A panel of recombinant inbred and congenic mice with various haplotypes, a series of transfected cell lines expressing chimeric H-2 K^d gene products and mouse anti-mouse, and rat anti-mouse H-2, and other lineage specific hybridoma antibodies were employed to determine the serological specificity of the hybridoma antibody (VMM-2), by FMF. Antibody reagents were either directly fluoresceinated or counter stained with a fluoresceinated Fab_2 portions of the appropriate second step antibody. One million cells from spleen, thymus , bone marrow or cultured cell lines were reacted with either directly labelled or culture supernatants containing antibodies followed by labelled second step antibody and tested for specific immunofluorescence by FMF.

To detect the VMM-2 binding antigen, lysates of T, B, and monocyte cell lines of $H\text{-}2^d$ haplotype were examined by Western analyses. Lysates were run either on a 8 or 14% PAGE-SDS (either reducing with 2-ME or non-reducing), either directly

stained with coumassie blue or transferred to Immobilon-P membrane, stained with the conventional anti-K^d and VMM-2 antibody followed by second step antibody coupled to alkaline phosphatase (ELISAmate staining kit from KPL).

Results:

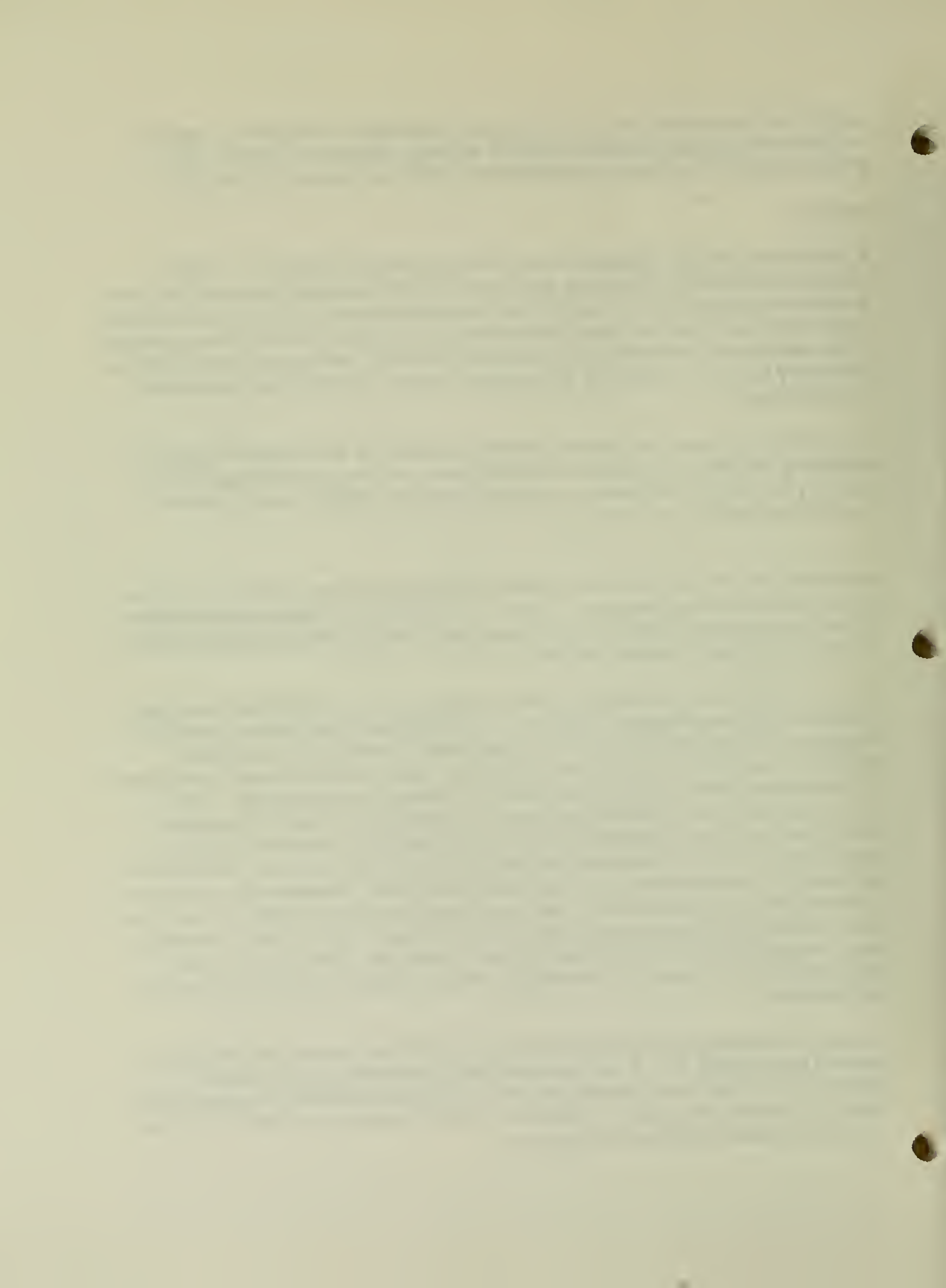
A hybridoma cell line secreting rat anti-mouse monoclonal antibody of $IgG2_b$ class was established. The tissue distribution by FMF analyses, showed that VMM-2 selectively stained cell populations from bone marrow and spleen while cells from lymph nodes and thymus were undetectably (dull) stained. the positive population in the spleen was further identified to be IgM^+ B cells. Where as T cells were not detectably stained. Further, the fluorescence intensity of the stained population was moderate.

Experiments to determine, whether VMM-2 is binding to an activation antigen, or circulating antigen or a cytoplasmic antigen was ruled out by unchanged binding intensity (by FMF). Additionally, moderate staining by VMM-2 was not due to weak binding affinity, as increased

concentrations of the antibody or prolonged incubation of the stained cells did not affect the fluorescence intensity. Further, the treatment of antigenic determinant by anti-glycosylating agents did not affect the binding intensity, suggesting VMM-2 may be specifically binding to a fine epitope.

The strain distribution of VMM-2 binding using a panel of recombinant inbred and congenic mice revealed that, VMM-2 is specifically binding to the cells from H-2 haplotype. In that, the cells from mice specifically carrying the K end the 'd' haplotype were found to be stained by VMM-2. Blocking experiments have further confirmed that VMM-2 specifically binds to a receptor on spleen cells. These results suggested that VMM-2 may be either binding to a unique H-2 antigen located at the K end of the MHC complex or recognizing a polymorphic form of class I gene product preferentially expressed on B cells of $H\text{-}2^d$ mice. Further, this antibody did not cross react with any other haplotypes. Analyses of transfected L cells expressing chimeric H-2 K^d gene products showed that VMM-2 binds to the cells expressing epitopes corresponding to the residues D152- S184 , located at the C terminal end of the $\alpha2$ domain of the K^d molecule. These results clearly prove that VMM-2 binds to a fine epitope which is located at the bottom part of the molecule.

Immunoblot analyses of lysates from lineage specific cell lines of $H\text{-}2^d$ haplotype showed that VMM-2 and a conventional anti-K^d antibody bind with similar intensity to a 45000 m.w. protein on all the cell types tested, in the order of B cells > T cells >I monocytes. Additionally, VMM-2 was found to stain a protein or proteins approximately of 200,000 m.w.



Significance:

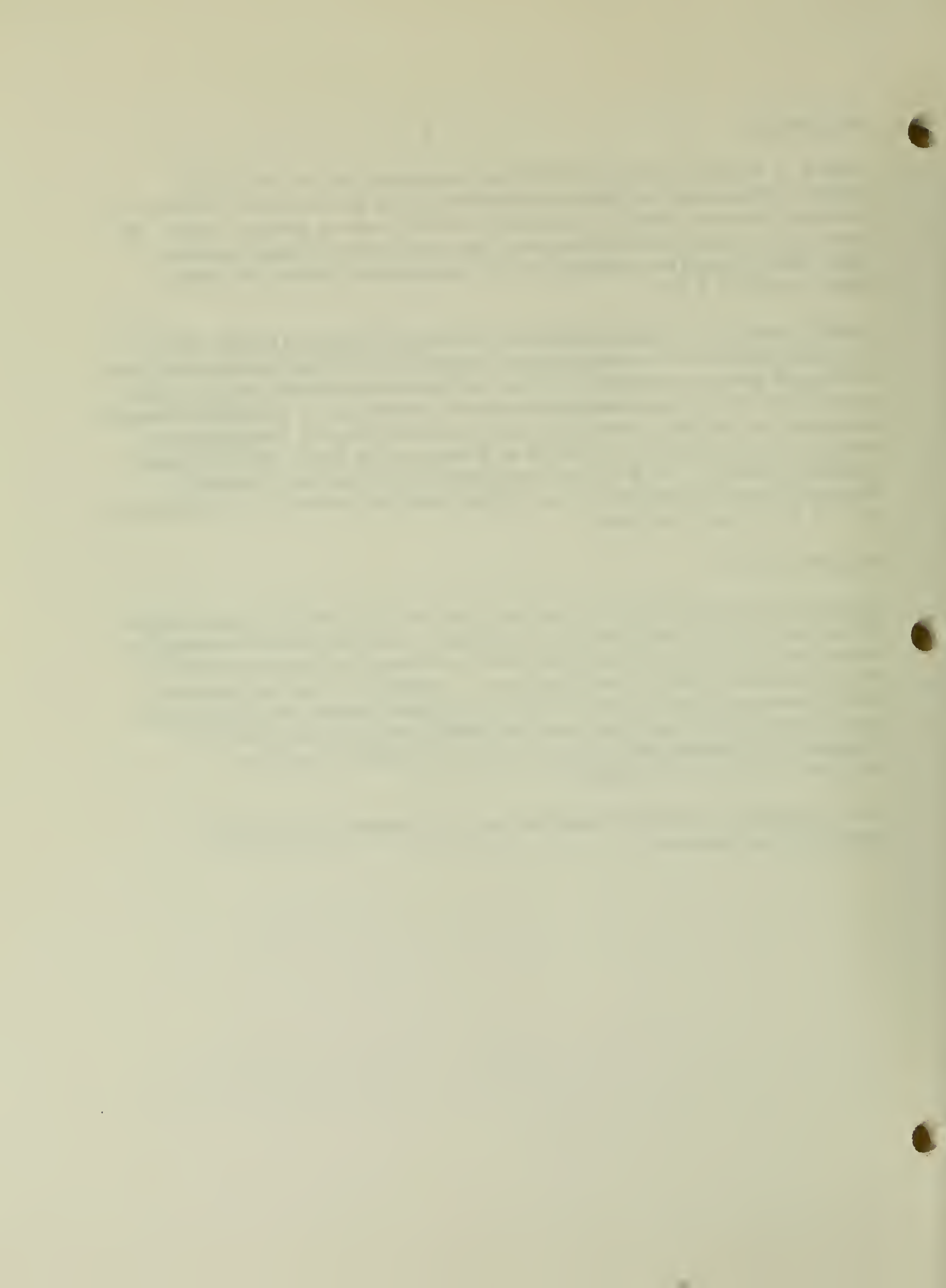
VMM-2 is an unique anti-H-2 K^d antibody recognizing one or two of the antigenic epitopes (out of 3 known)of described to be located on the $\alpha 2$ domain of the class I molecule, where T cell receptor of $CD\text{-}8^+$ cells is believed to bind. So far no antibodies recognizing this part of the molecule has not been reported. Hence VMM-2 could be a valuable tool for the studies on interaction between class I and $CD8^+$ T cells.

VMM- 2 uniquely recognizes a high molecular weight protein on B cells, which is not recognized by a conventional anti-K^d antibody. Recent reports show that class I molecules being polymorphic can occur as dimeric or tetrameric forms on T cells under certain physiological conditions such as viral infection or endogenous peptide processing. In the light of this evidence, it is conceivable, that high molecular weight protein recognized by VMM-2 on B cells may be those oligomeric cluster of class I molecules. As B cells are not involved in alloreactivity, the latter hypothesis may be applicable. Then, VMM-2 could be a valuable tool in dissecting the T-B interaction in apoptosis.

Proposed course:

Experiments are under way to further show that the high molecular weight protein stained by VMM-2 is really oligomeric molecule. In that, blots will be repeated to show that on a reducing gel polymeric forms will dissociate into monomers and all the binding by VMM-2 is at 45000 m.w. Additionally, the high molecular weight protein will be extracted, treated with reducing agents and detergents to gently separate monomers and show that VMM-2 still specifically binds to monomer, and the intensity staining on the western blot is due to the conglomeration of the monomers.

Upon completion of the above experiments it is anticipated to submit the manuscript for publication.



PROJECT NUMBER

BH 02017-02-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Regulation of arachiodonate metabolism at the level of protein synthesis

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

Thomas Hoffman, M.D., Chief Laboratory of Cell Biology
Crystal Lee, B.A., Biologist
Ezio Bonvini, M.D., Visiting Scientist
Elaine Lizzio, B.A., Microbiologist
Joseph Puri, Ph.D., Visiting Scientist
Anil Tripathi, M.D., Visiting Scientist

COOPERATING UNITS (if any)

NIH Blood Bank

LAB/BRANCH

Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

CBER/FDA NIH, Building 29, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Human monocytes treated with cycloheximide (CHX) demonstrated a dose-and-time dependent inhibition of PGE_2 synthesis and release in response to stimulation with PMA, iionomycin, serum-treated zymosan, or Con A. The effect of CHX required preincubation and was largely reversible within two hours. Thomboxane A_2 release was similarly affected but no comparable effects were observed on labeled arachidonic acid release or LTB_4 generation. The PGE_2 response in the presence of CHX was also inhibited when monocytes were given exogenous arachidonic acid with or without stimulation. CHX pretreatment also comparably decreased the amount of immunoreactive cyclooxygenase in resting and stimulated monocytes. These data indicate that monocyte cyclooxygenase, in contrast to phospholipase A_2 or 5-lipoxygenase and their regulatory proteins, turns over rapidly and may be a target for up-or-down-regulation by pharmacologic, or (potentially) physiologic agents which affect protein synthesis.

1. Objectives:

a. To understand the mechanisms underlying eicosanoid production and release.

b. To identify the which enzymes of arachidonate metabolism are regulatable a th level of translation

c. To establish a model for studies of the consequences of inhibition of products of the cyclooxygenase or lipoxygenase products in biological systems.

2. Methods employed:

a. Mononuclear cell isolation using ficoll-hypaque gradient separation of human peripheral blood obtained from volunteer donors through the NIH Clinical Center Blood Bank is combined with countercurrent centrifugal elutriation to obtain highly purified monocyte populations. Purity is evaluated by histochemical staining for monocyte associated enzymes such as alpha-naphthyl-acetate esterase in addition to Wright's staining.

b. Autocoid production is evaluated by immunoassay for prostaglandin E2 (PGE2) or Leukotriene B4 (LtB4). Alternatively, cells are labeled with 3H-arachidonic acid (AA*), and the radioactivity released into the supernatant analyzed by HPLC and liquid scintillation spectrophotometry using an in -line detector. In some instances, phospholipids inside the cell are analyzed by lipid extraction (Folch procedure) followed by thin layer chromatography and scanning.

c. Immunoblotting of cells lysates with antibodies directed against enzymes of the arachidonate cascade.

3. Major findings:

1) CHX treatment inhibited PGE2 and thromboxane B2 synthesis and release but had no effect of AA or LtB4 synthesis or release.

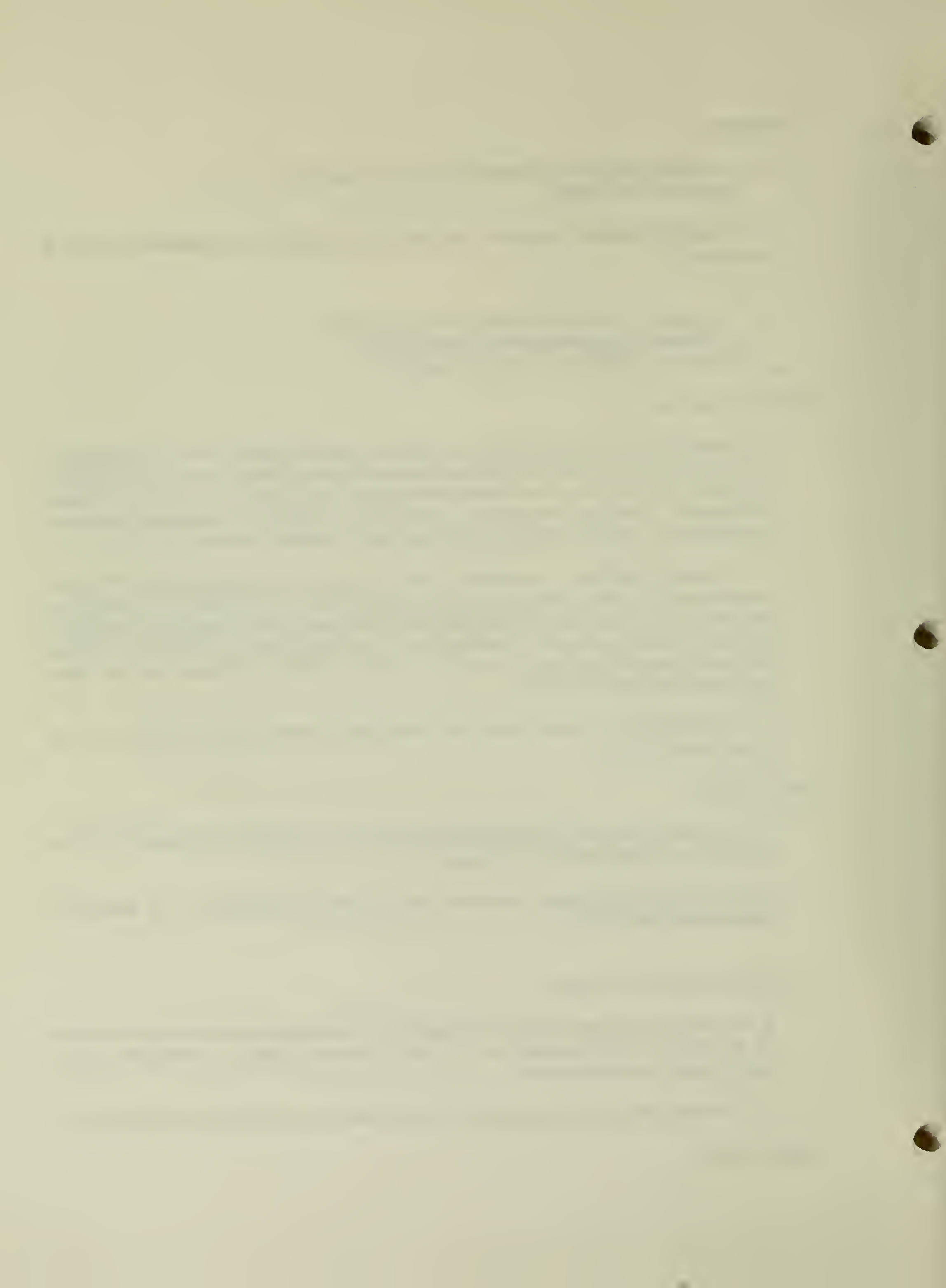
2) CHX treatment decreased the level of PGH synthase in monocytes in the absence of effects of the level of 5-LO.

4. Proposed course of the project:

a. The effect of cytokines and monoclonals directed against surface antigens of monocytes on transcription and translation of CO and LO enzyme genes in monocytes will be examined at the functional level.

b. The mechanism of gene regulation by cytokines will be examined at the molecular level.

5. Significance:



These studies represent fundamental research on monocyte function. Monocytes are important mediators of inflammation. A number of antibodies are now being promulgated to affect syndromes associated with septic shock and septicemia. These include murine and human anti-LPS antibodies. Of note is the effect of LPS on monocytes, in causing them to release mediators, including AA metabolites. PLA2 is also a component of the shock syndrome. By understanding its action we can better apply scientific principals to the regulation of these agents.

6. Publications.

1. Hoffman T, Lee YL, Lizzio EF, Tripathi AK, Bonvini E, and Puri J. Differential turnover of enzymes involved in human monocyte eicosanoid metabolism: cycloheximide treatment selectively inhibits cyclooxygenase product formation in the absence of effects on 5-lipoxygenase or phospholipase A_2. Biochem. Pharmacol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

BH-02022-01-LCB

PERIOD COVERED

May 30, 1992 through September 30, 1992

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

Immunogenicity of HIV-LPS-BA conjugates in SCID mice

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

Harm Snippe, Ph.D.
Thomas Hoffman, M.D., Chief, Laboratory of Cell Biology
Basil Golding, M.D.
Vijaya Manohar, Ph.D.
Elaine Lizzio, B.A.

COOPERATING UNITS (if any)

Eijkman-Winkler Laboratory for Medical Microbiology
Utrecht University
The Netherlands

LAB/BRANCH

Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

CBER, NIH, Building 29, Bethesda, MD

TOTAL STAFF 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.)

Only preliminary studies have been undertaken during the brief time the project has been underway. See proposal for details.

1. Objective

Severe combined immunodeficient (SCID) mice reconstituted with human lymphoid cells have inducible human immune function and may be useful as a small animal model for acquired immunodeficiency syndrome research.

2. Research proposal

To verify the literature, SCID mice will be reconstituted with human peripheral blood leukocytes (hu-PBL-SCID mice) and immunized with tetanus toxoid as a recall antigen and assessed for anti-tetanus antibody responses. (In principal hu-PBL-SCID mice do not respond to a primary immunization, but will respond to tetanus toxoid). A separate group of mice will be immunized with a trinitrophenyl-lipopolysaccharide-Brucella abortus conjugate (TNP-LPS-BA), a peculiar antigen which is known to behave as a thymus independent (TI) antigen and induces IgM responses in both normal and nude mice. The expectation is that unprimed human cells (as present in hu-PBL-SCID mice) will respond to this type of antigen with a primary anti-TNP antibody response. Thereafter, experiments will be performed with a HIV-peptide-LPS-BA conjugate and the sera of these mice will be tested for anti-peptide and anti-HIV antibodies and for neutralization capacity.
In separate groups of experiments elutriated cells will be used. These cell preparations contain 3-5 % stem cells. The cells will be injected intraperitoneal into SCID mice and immunized with tetanus toxoid, TNP-LPS-BA or HIV-LPS-BA. The number of CD34 positive cells is determined by FACS analysis before injection and at time intervals during the experiment.
After the initially experiments, several approaches will be followed to enrich the number of stem cells and the maturation of these cells into mature B-cells with the capacity to respond to the indicated antigens:
In vivo the mice will be treated with Human Stem Cell factor (HSF) and IL-7 for a couple of weeks, followed by IL-4 and IL-6 before antigen is given.
In vitro stem cells will be purified by adhesion to CD34 coated columns (CellProbe) and cultivated in vitro using the same interleukins as stated above. In addition IL-1, IL-3 and Leukemia Inhibitory Factor (LIF) will be tested for their effectiveness to develop stem cells into the B-cell lineage. FACS analysis and probes will be the major tools to control the process.
New antigen preparations (HIV-peptides) and adjuvant formulations (low toxicity, e.g. Q-Vac, Nonionic Block Polymers) will also be tested in this protocol.

2. Significance:

These studies are essential in order to develop a new generation of peptide based vaccines. The results obtained are general applicable because the study is based on fundamentals in immunology. Hopefully these studies will result in for instance a safe and effective HIV vaccine.

3. Publications:

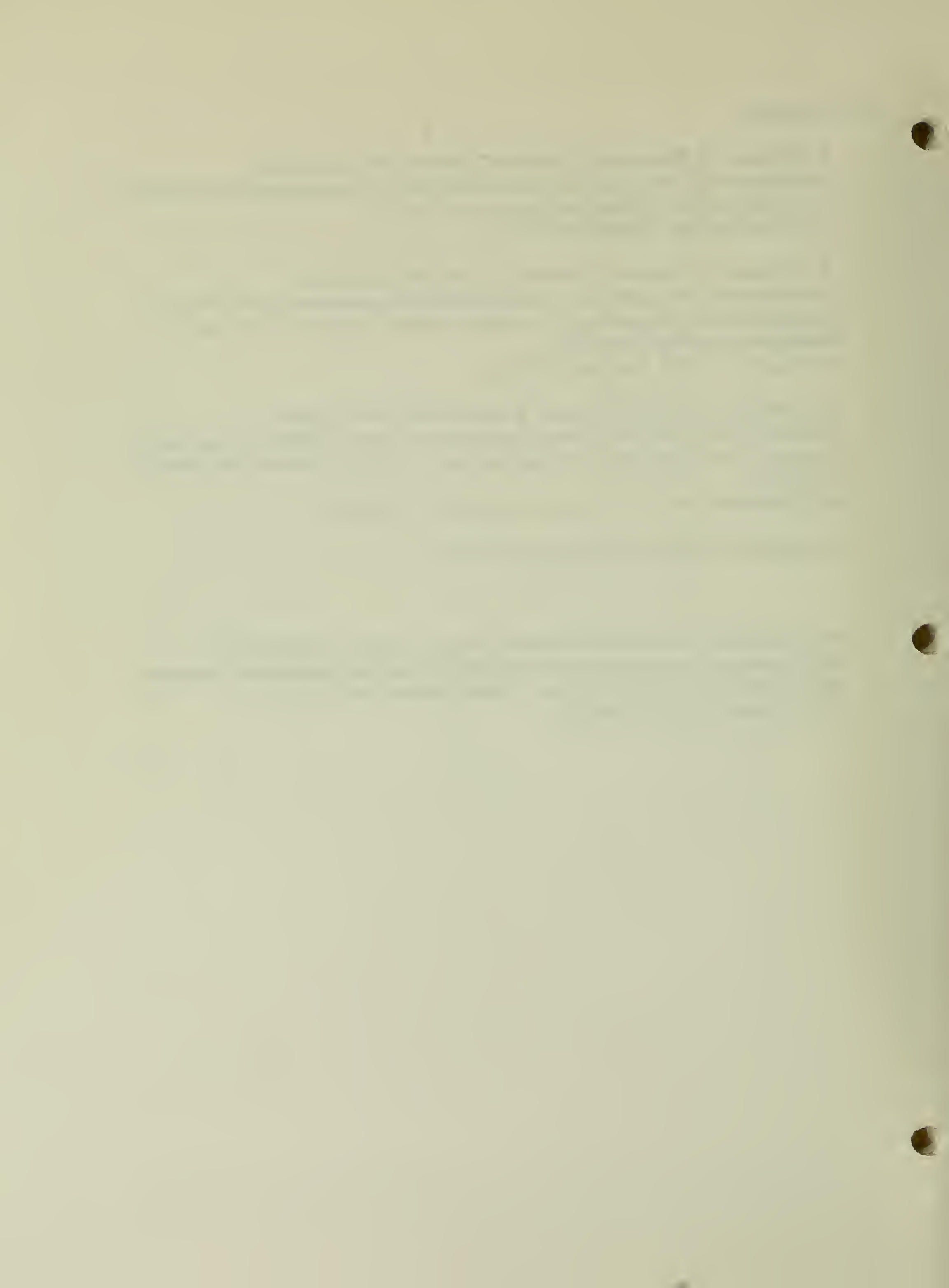
1. Goldstein J; Hernandez D; Frasch C; Beining PR; and others
Immunogenicity of lipopolysaccharide derived from Brucella abortus: potential as a carrier in development of vaccines for AIDS.
Adv Exp Med Biol 1991;303:227-33

2. Goldstein J; Hoffman T; Frasch C; Lizzio EF; and others
Lipopolysaccharide (LPS) from Brucella abortus is less toxic than that from Escherichia coli, suggesting the possible use of B. abortus or LPS from B. abortus as a carrier in vaccines.
Infect Immun 1992 Apr;60(4):1385-9

3. Golding B; Golding H; Preston S; Hernandez D; and others
Production of a novel antigen by conjugation of HIV-1 to Brucella abortus: studies of immunogenicity, isotype analysis, T-cell dependency, and syncytia inhibition.
AIDS research and human Retroviruses 1991 (7):435-46

4. Manohar, V; Brown,E.M.;Chused, T.M.;

Murine splenic null cell compartment contains distinct haemopoietic subpopulations: enlargement of a myeloid and an undifferentiated subset with the development of splenomegaly in New Zealand black mice.
Immunology 1992 75, 488-55



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02008-03-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Development of T-Independent Vaccines for HIV-1 Infection

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

B. Golding, Senior Investigator

M. Betts, Lab Tech (Bio)

M. Brunswick, Staff Fellow

T. Hoffman, Lab Chief

COOPERATING UNITS (if any)

Laboratory of Immunology, NIAID, NIH (J. Inman); RIBI, Immunochem. Corp. (K Myers)
Laboratory of DNA Viruses, Div. of Virology, CBER, FDA (H. Golding)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

CBER, FDA, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.7	3.5	0.2

CHECK APPROPRIATE BOX(ES)

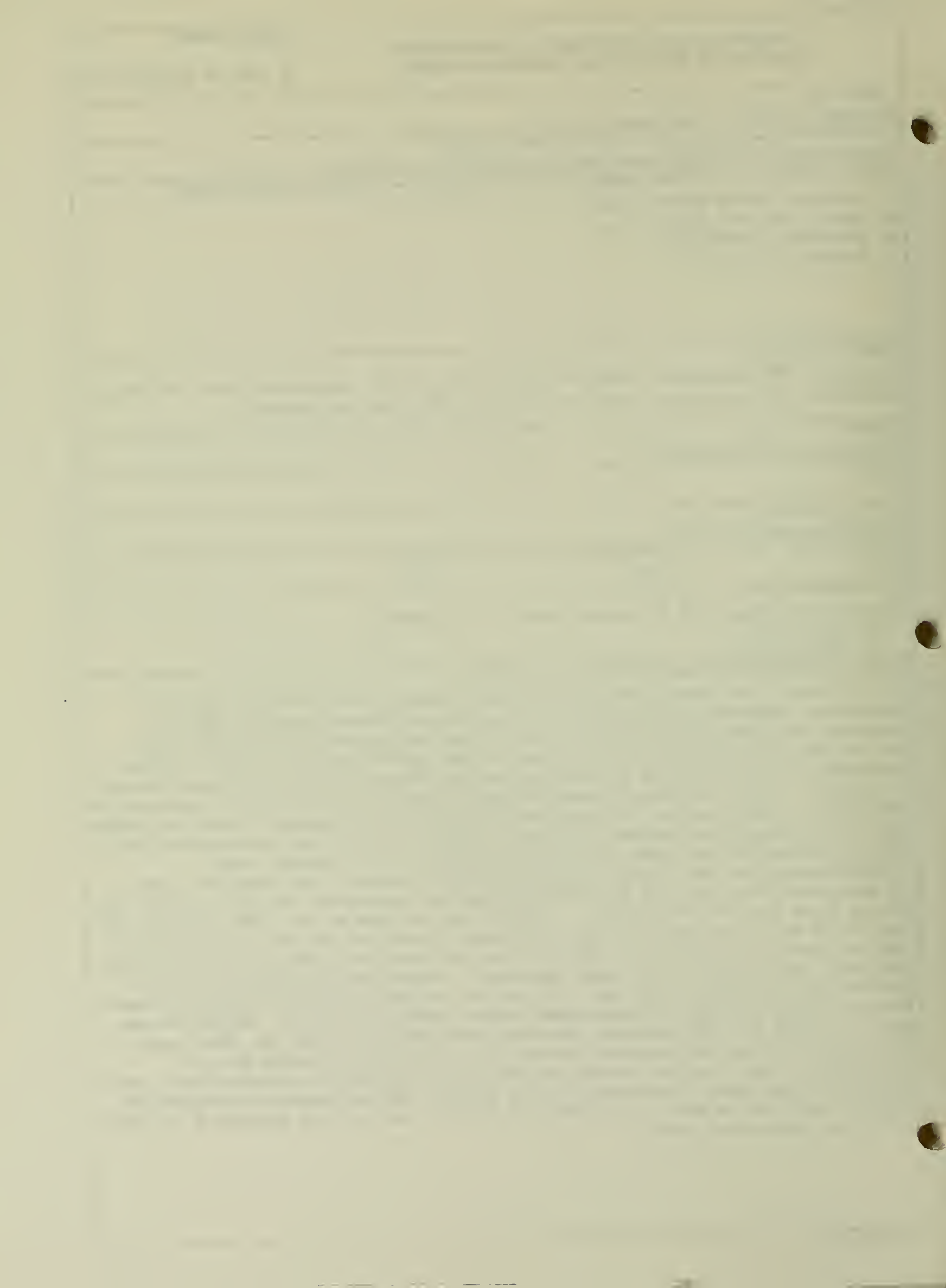
☐ (a) Human subjects ☑ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

HIV-1 infection is associated with decreased T helper cell function, so that vaccines or immunotherapy designed to stimulate the immune system of persons harboring the AIDS virus need to circumvent the requirement for T helper cells. Our strategy to accomplish this has been to use immune carriers which are T-cell independent and are capable of inducing antibody responses in the relative absence of T cells. Heat inactivated Brucella abortus (BA), has been shown to behave as T-independent type 1 carrier in human and murine antibody responses. Thus, we showed that HIV 1 proteins conjugated to BA were capable of eliciting neutralizing anti-HIV antibodies in mice, even in the relative absence of T cells. Sine lipopolysaccharide (LPS) from other gram negative bacteria can stimulate B cells, we postulated that LPS from BA would have similar properties. We purified LPS from BA and first test it for toxicity. Compared to LPS from E. coli (EC) LPS from BA was 10,000 fold less potent in inducing fever in rabbits; 300 fold less potent in causing lethality in mice; and 300 fold and 1400 fold less potent in inducing 1L1B and TNFa, respectively, from human monocytes. These results suggested that LPS from BA was much less likely than LPS from EC, to induce endotoxic shock in humans. We then tested LPS from BA from immune carrier function in mice. TNP-LPS BA was able to induce anti-TNP antibody repsonses from BALB/c, athymic and CBA/N mice. Thus, LPS BA, like the bacterium from which it is derived, behaves as a T-indenpendent type 1 carrier and may be useful as a carrier for vaccines which could bypass the requirement for helper T cells in HIV-1 dervied peptides conugated to Brucella abortus for ability to induce anti-HIV-1 neutralizing antibodies in mice and in the SCID-human model.



Project 1:

Title:

Development of T-independent Vaccines for HIV-1 Infection

Investigators:

Basil Golding, LCB.
Others in LCB: Diana Hernandez, Paul Beining, M. Betts,
E. Lizzio, J. Goldstein, and T. Hoffman.
Division of Virology, CBER, FDA: H. Golding.
NIAID, NIH: J. Inman.

Rationale:

HIV-1 infection precedes the development of AIDS by several years and is associated with impaired CD4+/helper T cell function and numbers. In order to boost immune responses in persons with asymptomatic HIV-1 infection it is probably necessary to bypass the requirement for helper T cells. BA has been shown to stimulate mouse and human B cells in the relative absence of T cells and is therefore being tested as a carrier for HIV-1 and HIV-1-derived peptides. In attempting to refine the carrier, it is important to identify the cell wall component of BA that manifests BA immune activity and is free of other molecules which may induce unwanted effects. Since LPS from other gram negative bacteria have been shown to be very active in stimulating B cells, LPS from BA was a likely candidate as a cell wall component that would possess similar immune characteristics as the whole bacterium.

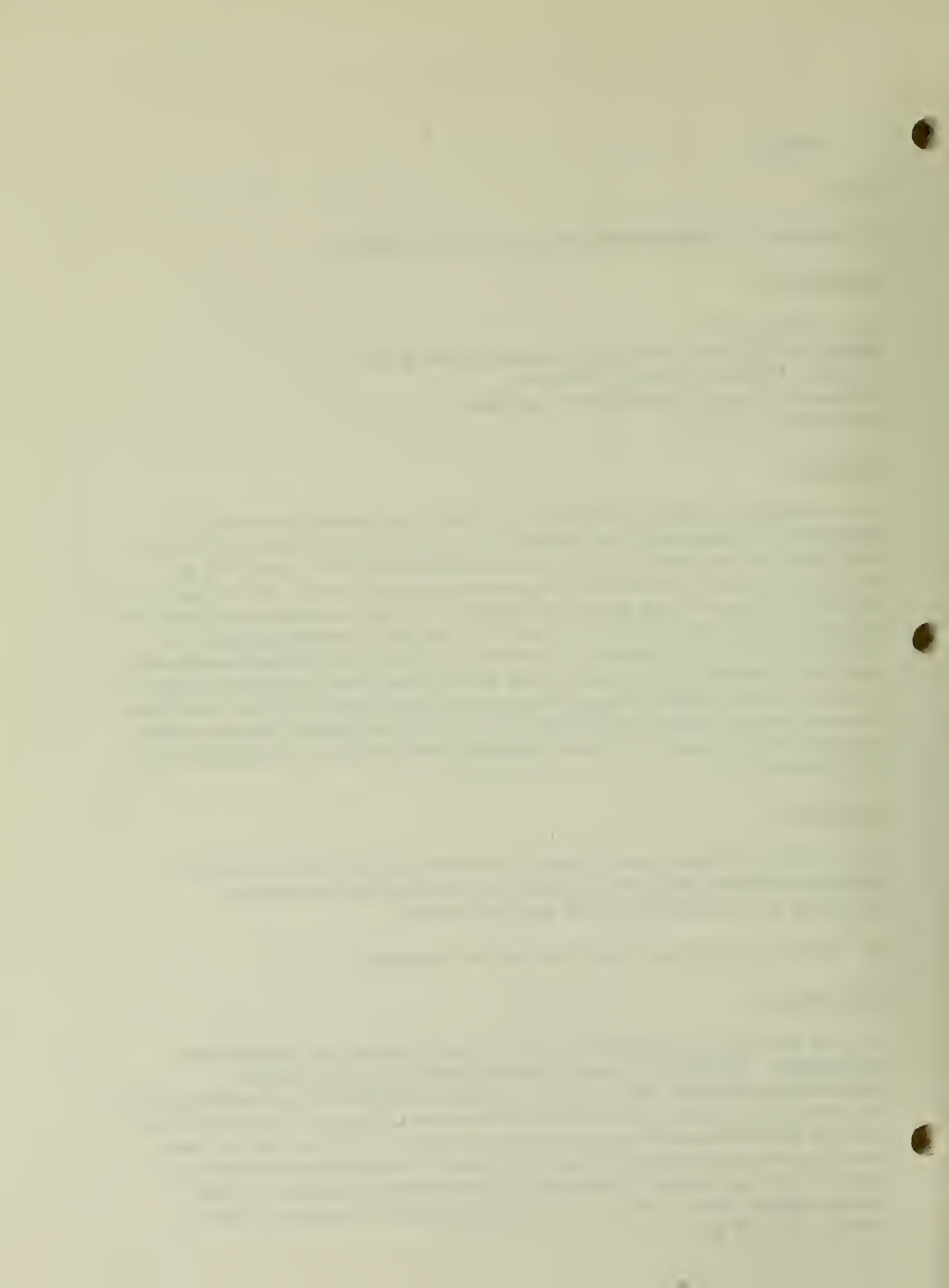
Objectives:

(1) To identify peptides within the HIV-1 envelope (gp120) which are likely to generate neutralizing antibodies against HIV-1 and to determine whether conjugates of these peptides to BA are immunogenic.

(2) Testing of LPS BA as a carrier for antibody responses.

Major findings:

(1) LPS from BA was purified by butanol-water extraction and characterized biochemically. SDS-PAGE showed a pattern typical for LPS BA and the ketodeoxyoctanate was 1% by weight. Protein and nucleic acid contamination was less than 2% by weight. This LPS was compared to LPS from E. coli (LPS EC) and shown to be 10,000-fold less potent in inducing fever in rabbits, 268-fold less potent in killing D-galactosamine treated mice and 1,400-fold and 400-fold less potent in eliciting release of TNFα and IL1β from human monocytes. These findings suggest that LPS BA is much less likely to evoke endotoxic shock in humans than LPS EC.



(2) LPS from BA was haptenated with trinitrophenyl (TNP) and used to immunize BALB/c, athymic, CBA/N, and C3H/HeJ mice. The IgM and IgG subclass anti-TNP responses in these mice were compared to those obtained with TNP-KLH (prototype T-cell-dependent, TD); TNP-BA and TNP-LPS EC (T-independent type 1, TI-

1); and TNP-ficoll (T-independent type 2, TI-2). LPS BA provided carrier function for TNP and induced anti-TNP IgM and all IgG subclasses in BALB/c mice. Furthermore, similar to other T-independent antigens (TI-1 and TI-2), TNP-LPS BA elicited anti-TNP antibody responses in athymic mice. TNP-LPS BA, unlike TNP-ficoll, also induced responses in CBA/N mice. Taken together, these results indicate that LPS BA can substitute for the BA bacterium as a carrier and that it behaves as a TI-1 antigen.

3) A peptide was synthesized consisting of the GPGRAF sequence from the V3 loop of HIV-1 envelope which was flanked by glycine and alanine residues. This construct was linked to BA. The conjugate behaved as a T-independent type 1 antigen in mice, since anti-peptide responses were observed in BALB/c and CBA/N mice. Furthermore, these antibodies could inhibit gp120/CD4 mediated-syncytia, albeit at a relatively low titer.

Future goals:

(1) Longer peptide constructs from the V3 loop as well as gp120 (produced in CHO cells) will be used in order to increase the titer of neutralizing antibodies and increase the likelihood of generating antibodies which recognize conformational determinants.

(2) Antibodies generated by peptide-BA conjugates will be tested for ability to neutralize diverse HIV-1 isolates.

(3) In future experiments the peptide-BA antigens will be tested in primates and/or in the SCID-human model for immunogenicity and for ability to generate antibodies which neutralize virus in vitro and in vivo.

Publications on this project:

Golding B, Golding H, Preston S, Hernandez D, Beining PR, Manischewitz J, Harvath L, Blackburn R, Lizzio E, and Hoffman T. Production of a novel antigen by conjugation of HIV-1 to *Brucella abortus:* studies of immunogenicity, isotype analysis, T-dependency, and syncytia formation. AIDS Research and Human Retroviruses 1991;7:435-446.

Goldstein J, Blay R, Frasch C, Beining PR, Betts M, Hernandez D, Hoffman T, and Golding B. In: Atassi MZ ed. Advances in Experimental Medicine and Biology. Immunogenicity of Brucella abortus, and lipopolysaccharide derived from Brucella abortus, in mouse and human: Potential as carriers in development of vaccine for AIDS. Plenum Publishing Corp., New York. 1991.

Goldstein J, Hoffman T, Frasch C, Lizzio EF, Beining PR, Hochstein DR, Lee YL, Angus RD, and Golding B. Lipopolysaccharide from *Brucella abortus* is less toxic than lipopolysaccharide from *E. coli,* suggesting the possible use of *B. abortus* as a carrier in vaccines. Infection and Immunity, 1992;60:1385-1389.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02009-03-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

TH1-Type Responses by Human T Cells Stimulated by Brucella abortus 009-03

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

B. Golding, Senior Investigator
M. Betts, Lab Tech (Bio)
T. Hoffman, Lab Chief

COOPERATING UNITS (if any)

Lab of DNA Viruses, Div of Virology, CBER, FDA (M. Zaitzeva, J. Quan, H. Golding)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

CBER, FDA, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.9	3.1	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.)

Since we are considering Brucella abortus (BA) as a carrier for human vaccines, especially for HIV-1 infected persons, it was important to know what effect if had on human T cells. Evidence from murine studies, performed in vivo, suggested that repeated BA injections caused interferon-gamma (IFNy) release and expansion of TH1 cells. We found that human T cells (85-95% CD3+ by flow cytometry) secred IFNy in response to BA and lipopolysaccharide (LPS) from BA. This response to BA was increased synergistically in the presence of IL-2. Both CD4+ and CD8+ T cells, separated by panning, released IFNy when stimulated by BA. T cells from HIV-1 infected asympotmatic persons were also able to respond to BA and secrete IFNy. However, T cells from patients with symptons were only responsive if IL-2 was present. IFNy has been shown to be a product of TH1 cells, IL-4 secretion, which is a function of TH2 cells, was assessed using a cell line (CtH4), cells stimulated by BA did not induce proliferation of CtH4, which was sensitive to the presence of 10 pg/ml of IL-4. Similarily, no IL-4 was detected in these supernatants in the ELISA, which was sensitive to 30 pg/ml of IL-4. These results were confirmed by PCR analysis, which showed that BA induced IFNy, but not IL-4 mRNA. These data suggest that BA LPS from BA are capable of selectively stimulating human TH1 cells. This has important implications in vaccine development since a TH1 cell bias may afford greater protection against certain infections including HIV-1.

Project 2:

Title:

TH1-Type Responses by Human T Cells Stimulated by *Brucella abortus*.

Investigators:

Basil Golding, LCB.
Others in LCB: M. Betts, R. Blay, and T. Hoffman.

Rationale:

It has been shown that repeated injection of mice with BA results in IgG2a being the predominant IgG isotype (Finkelman et al). Switching from IgM to IgG2a was shown to depend on the presence of IFNγ (Snapper and Paul). Furthermore T cells plated at limiting dilution following repeated in vivo exposure to BA were mainly of the TH1 phenotype, i.e. they secreted IFNγ and IL2 but not TH2 products such as IL4 (Mossman et al). These data all pointed to the likelihood that BA stimulated TH1 cells directly to release IFNγ. It was important to test this possibility in human cells because we are developing BA as a candidate carrier for human vaccines (e.g. for HIV-1 infected persons) and IFNγ has antiviral effects as well as multiple effects on human immune cells. These latter effects may be important in resisting infection. TH1 cells appear to play a protective role in several murine and human diseases, possibly due to IFNγ secretion or via other mechanisms.

Objectives:

(1) To determine whether human T cells (CD4+ and/or CD8+) secrete cytokines (IFNγ and /or IL4) in response to BA and LPS BA.

(2) To determine whether T cells from HIV-1-infected persons can respond to BA in terms of IFNγ release.

(3) To determine whether BA or LPS BA could activate TH1 cells.

Major findings:

(1) BA and LPS BA induced IFNγ secretion from normal human CD3 and T cells. Both CD4+ and CD8+ T cells responded. This response was increased in a synergistic manner when IL2 was added to the cultures.

(2) BA could also induce IFNγ release from T cells obtained from asymptomatic HIV-infected persons. However, T cells from symptomatic HIV-infected persons only responded when IL2 was present in the cultures.

(3) BA and LPS BA were incapable of eliciting detectable IL4 secretion from human T cells. Supernatants from the cultures were tested in a functional assay (which measures proliferation of a mouse cell line transfected with human IL4 receptor) sensitive to 4 pg/ml of IL4 and in an ELISA sensitive to 30 pg/ml of IL4.

Significance:

(1) The finding that BA and LPS BA have TH1-stimulating activity has implications for use of BA and LPS BA as carriers for vaccines. Induction of IFNγ would be beneficial in enhancing immunity against intracellular pathogens by stimulation of increased MHC gene expression; increased NK, macrophage and cytotoxic T cell killing, and increased differentiation of antibody secreting B cells. In addition, IFNγ has antiviral activity.

(2) There are several disease models in humans and mice, in which increased susceptibility to infection is associated with a bias towards TH2 cells. BA and LPS BA used as vaccines/immunotherapeutic agents in these diseases may reverse the balance in favor of TH1 cells.

(3) Allergic diseases are associated with elevated IgE levels due to IL4 secretion. It may be possible to counter this effect by using BA or LPS BA linked to appropriate epitopes to stimulate TH1 cells. The resultant release of IFNγ should down-regulate IL4 production and reduce switching to IgE.

Future goals:

(1) Confirm the effect of BA and LPS BA at the mRNA level by PCR using oligonucleotide primers specific for IFNγ and IL4.

(2) Use BA and LPS BA as stimuli of IFNγ to determine which pathways are involved in IFNγ gene activation. In preliminary experiments increased nuclear translocation of NFKb and AP-1 has been observed.

(3) Determine, at the protein and mRNA levels, whether BA or LPS BA can down-regulate production of IL4 by T cells stimulated by αCD3 and by allergens.

(4) Determine whether triggering of T cells by BA and LPS BA involves the T cell receptor (TCR) which interacts with the MHC-peptide complex.

Publications on this project:

Blay R., Hernandez D, Betts M, Clerici M, Lucey D, Hendrix C, Hoffman T, and Golding B. *Brucella abortus* stimulates human T cells from uninfected and infected individuals to secrete IFNg. AIDS Research and Human Retroviruses, 1992;8:479-486.

Golding, B. Cytokine regulation of humoral immune responses. In: Spriggs DR and Koff WC eds. Topics in vaccine adjuvant research CRC, Boca Raton, FL, 1991;45-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 02023-01-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Molecular Mechanisms of T Lymphocyte Activation

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

Ezio Bonvini, Visiting Scientist, LCB, DH, CBER
Karen E. Debell, Microbiologist, LCB, DH, CBER
Antonio Conti, Visiting Fellow, LCB, DH, CBER
Jorge Laborda, Visiting Scientist, LCB, DH, CBER

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Division of Hematology

INSTITUTE AND LOCATION

Center for Biologics Evaluation and Research, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The objective of this study is to investigate the coupling mechansism between the T cell receptor (TCR)/CD3 complex of lymphocytes and phospholipase C (PLC), the key enzyme in the inositol phospholipid (InsPL) hydrolysis pathway. This pathway generates second messengers that may be critical in inducing T cell activation. An understanding of the mechanism of lymphocyte activation may offer a basis for designing immunomodulatory strategies targeted to critical elements of the signal transducion pathway. A strategy of cell permeabilization has been developed in our laboratory which allows access to micromolecules (e.g., nucleotide, peptides, etc.) to the intracellular enviromnment of lymphocytes, while maintaining coupling of the TCR/CD3 complex with PLC. By using this strategy, we have obtained information on the regulation of InsPL hydrolysis and on the stoichiometry of the assoication of a _src_ kinase, _fyn_, with polypetides of the CD3 complex. By the use of this strategy, we will seek information on the mechanism of control of PLC activity. Coprecipitation using anti-PLC Ab and _src_-family products (_fyn, lck.yes,_ etc.) will be used. Transient or "weak" interactions will be "stabilized" by using homobifunctional chemical cross-linker in permeabilized cells followed by immunoprecipitation. This strategy was previously used successfully to obtain stoichionetry data of CD3/_fyn_ interation. By using inhibitors of protein tyrosine phosphate phosphatase (including peptides which may function as potential competitive inhibitors, such as phosphorylated _fyn_ or _lck_ peptides) in intact and permeabilized cells, attention will be paid to the role of these enzymes, including the CD45 phosphatase, in modulating insPL hydrolysis. Since previous evidence suggests heterogeneity in signal transduction among different T cell subsets, attention will be paid to potential differences and their functional implications. AN initial attempt will be made to construct T cell clones defective in PLC (PLC-γ1) by genetically "knocking out" the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-02024-01-LCB

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Regulation of Phospholipase C Activation and Inositol Phosphate Metabolism

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

Antonio Conti, Visiting Fellow, LCB, DH, CBER
Ezio Bonvini, Visting Scientist, LCB, DH, CBER
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COOPERATING UNITS (if any)

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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.)

Perturbation of the T cell receptor (TCR)/CD3 complex by anti-receptor antibodies (Ab) mimcs antigen activation and stimulates an inositol phospholipid (InsPL) - specific phospholipase C (PLC) resulting in generation of second messengers. Of these metabolites, inositol 1,4,5)P_4) control Ca^{2+} mobilization. This project addresses the role of intracellular Ca^{2+} in regulating this pathway. Murine T cells permeabilized with a bacterial lysin are used as a model. Intracellular free [Ca^{2+}] is controlled by a calcium/magnesium/EGTA buffer. Aggregation of CD3 complexes with the combination of a specific monoclonal Ab (mAb) and a second Ab induced inositol phosphate accumulation in the absence of added Ca^{2+}. CD3-induced InsPL hydrolysis increased with the free Ca^{2+} concentration reaching a maximum at 100-300 nM [Ca^{2+}] and decrease thereafter. Increasing free [Ca^{2+}] to 300 nM increased the overall efficiency of hydrolysis, without changing the affinities governing CD3 coupling to PLC. Permeabilized cell's response to CD3 aggregation diminished, due to a mixed type inhibition, when exposed to >300 nM free [Ca^{2+}]. Ca^{2+} alone had no effect on inositol phosphate levels in permeabilized cells. Only polyphosphoinositides were cleaved, irrespective of the Ca^{2+} concentration. No accumulation of Ins(1)P/Ins(3)P was detected, indicating that direct hydrolysis of phosphatidylinositol did not occur. Free [Ca^{2+}] above 300 nM shifted the relative levels of CD3-induced Ins(1,4,5) P_3 and Ins (1,3,4,5) P_4 accumulation in favor of the latter, and enhanced the conversion of authentic [^3H] Ins (1,4,5)P_3 to Ins (1,3,4,5)P_4. These data suggest that, although free Ca^{2+} not requried, InsPL hydrolysis is optimally triggered by CD3 perturbation at intracellular Ca^{2+} levels approximating those observed in intact resting lymphocytes (100 nM). Ca^{2+} concentrations of a magnitude similar to those triggered in intact cells by InsPL-derived metabolites may negatively affect InsPL hydrolysis and promote Ins (1,3,4,5) P_4 production, thus controlling the intracellular amounts of Ins (1,4,5)P_3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-02025-01-LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Role of Cytoskeleton Microfilament Assembly in TCR/CD3 Signal Transduction

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

Karen E. Debell, Microbiologist, LCB, DH, CBER
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Antõñio Conti, Visiting Fellow, LCB, DH, CBER

COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

CHECK APPROPRIATE BOX(ES)

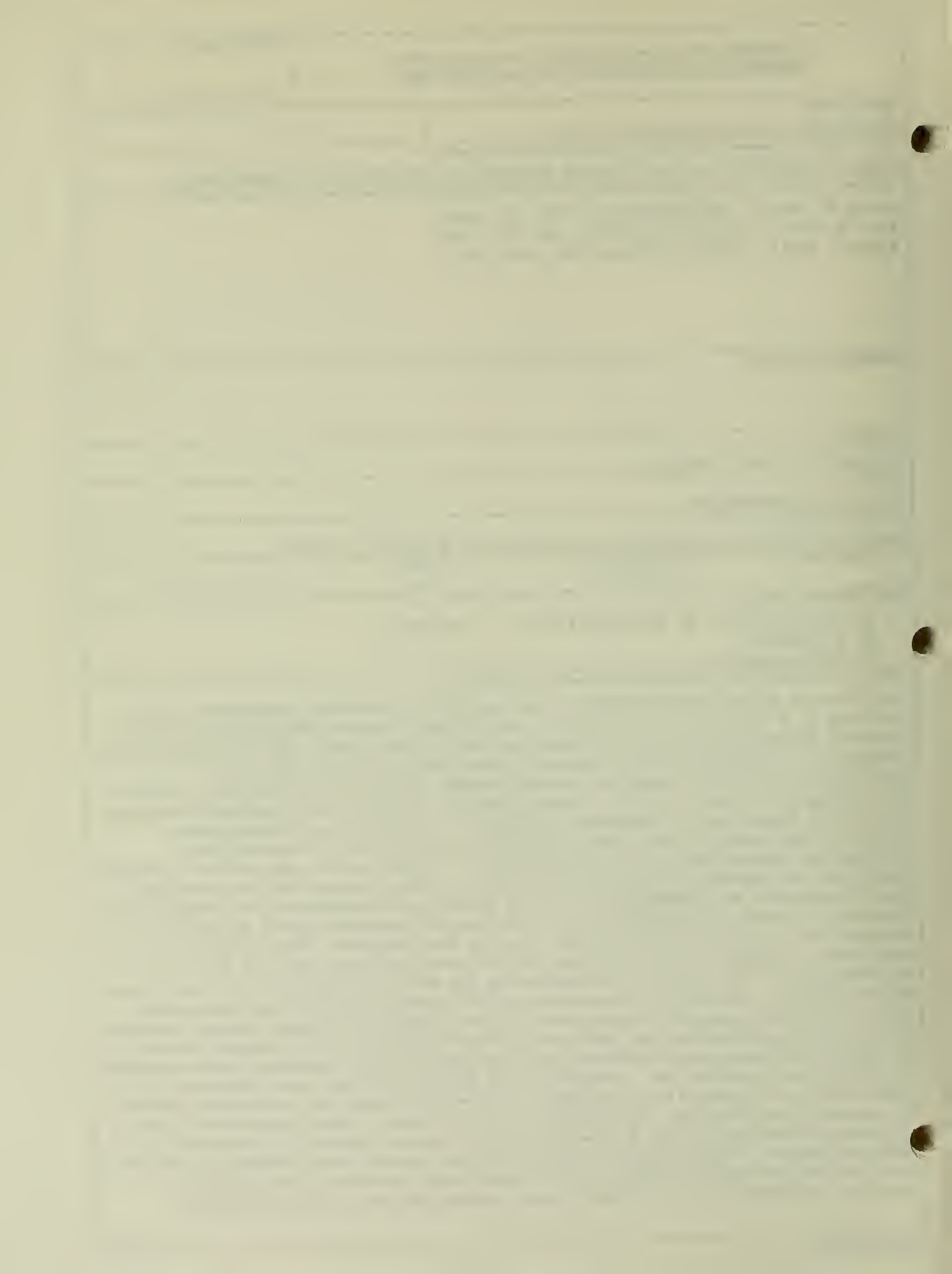
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Perturbation of the TCR/CD3 complex with anti-TCR or anti-CD3 antibodies (Ab) is followed by a sequence of biochemical and biological responses similar to those observed subsequent to T-cell interaction with antigen (Ag). We have reported that assembly of cytoskeletal microfilaments occur rapidly in response to Ab perturbation of the CD3 complex. Microfilaments are imortant structural cellular elements and may have a role in internalization, routing, and, possibly, coupling of surface recepotrs with effector mechanisms. Disruption of microfilament assembly with specific inhibitors (cytochalasins) may have functional and biochemical consequences on T cell activation. Cytochalas in pretreatment enhanced hydrolysis of membrane inositol phospholipids (InsPL) induced CD3 perturbatiobn. InsPL hydrolysis by phospholipase C (PLC) generates second messengers which may be involved in signal transduction. Potentiation of InsPL hydrolysis by cytochalasins affected the initial rate and maxima level of the response. Decay rates were unaffected, suggesting that the turn off mechanism is independent of the microskeleton. The effect of cytochalasins did not correlate with the association of the TCR/CD3 complex with detrgent-insoluble cytoskeleton, but was associated with a decreased receptor internalization rate. The relationship between recepotr turnover and internalization, and signal transduction was characterized in detail. Ab immobilized onto solid matrices have been used as an alternative to perturbation of TCR/CD3 with soluble Ab. Conditions and requirements for the interaction of T lymphocytes with immobilized anti-TCR or anti-CD3 Ab have been previously defined. As opposed to soluble Ab, the rigidity of a solid matrix would effectively prevent potentiate InsPL hydrolysis even when receptor internalization is prevented by physical means. This observation indicates that microfilament disruption may favor coupling of the TCR/CD3 complex to the signallying apparatus, and suggests a negative feed-back role for microfilament polymerization in PLC activation.



PROJECT OVERVIEW

OBJECTIVE

To analyze and characterize the biological response and the underlying biochemical mechanisms in the induction of specific T lymphocyte functions in response to defined ligand/receptor interaction, including the interaction of MoAb with surface molecules.

RATIONALE

T lymphocytes are essential elements of the immune response. A coordinated immune response depends on a network of interactions between T cells, B cells and "accessory" cells (monocytes/macrophages, dendritic cells, etc.). The $CD4^+$, T helper cell subset (Th) of T lymphocytes is a key element of this response. The immunological consequences of selective depletion of $CD4^+$ cells is central to the clinical picture of the Acquired Immune Deficiency Syndrome (AIDS).

This network of interactions is regulated by a sophisticated set of intercellular mediators (cytokines) and, under certain circumstances, requires cell-cell contact. This direct cellular interaction is mediated by specific "contact" molecules (cell adeshion molecules) and receptors.

Key to the initiation of the immune response is the recognition of antigen (Ag) by the T cell antigen receptor (TCR). The TCR (murine or human) is comprised of an Ag-specific, variable (idiotypic) portion (Ti) and a non-variable complex (CD3). The mature TCR is composed of two polypeptide chains (α and β or γ and δ) which are non-covalently associated with the CD3 complex. Several polypeptide chains form the CD3 complex, all of which are membrane associated and span the cell cytoplasm, but have only small exracellular domains. The CD3 is probably the signal transducing portion of the TCR. The TCR recognizes processed Ag in the context of the major histocompatibility complex (MHC) of Ag-presenting cells (APC). $CD4^+$ cells recognize Ag in the context of MHC class II molecules, while $CD8^+$ cells recognize MHC class I molecules. This cell/cell interaction is mediated by both direct recognition of the Ag/MHC complex by the Ti portion of the TCR and CD4 (or CD8) interaction with the MHC.

TCR perturbation by treatment with Ab directed against its component (either Ti or CD3) induces biochemical and biological responses which resemble those provoked by Ag stimulation, but in the absence of accessory cells. Ab-mediated perturbation of the TCR is a useful tool for dissecting funtional association with its component. In addition, these Ab may constitute a basis for attempting pharmacological modulation of T cell function.

In the mouse, Th cells may be divided into two, or possibly more, different subpopulations based on genotypical, phenotypical and functional characteristics. Th type I cells (Th1) produce IL2 and interferon$-\gamma$, while Th type II cells (Th2) produce IL4 and other lymphokines, including IL1. Th1 cells are thought to be implicated in delayed type hypersensitivity, while Th2 cells are involved in helper function in the context of Ab production. This distinction has now been established also for human T lymphocytes.

TCR stimulation is followed by a variety of biochemical changes. Activation of the adenylyl cyclase (AC) with production of adenosine 3'-5'(cyclic) monophosphate (cAMP), mobilization of Ca^{2+} from either intracellular or extracellular compartments, activation of protein serine- and threonine-specific protein kinases (such as Ca^{2+}- and phospholipid-dependent protein kinase (PKC), Ca^{2+}/calmodulin-dependent protein kinase, and cAMP-dependent protein kinase (PKA)) as well as of tyrosine-sepcific protein kinases, with phosphorylation of a variety of cellular proteins (among which the ζ chain of the CD3 complex and a phospholipase C (PLC) isozyme, PLC$-\gamma 1$) have all been reported. Ca^{2+} mobilization and PKC activation have received particular attention, since mimicking of Ag-induced T cell activation could be accomplished by treating cells with phorbol ester activators of PKC (such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA)) and Ca^{2+} ionophores (i.e.: ionomycin).

A unique metabolic pathway, the hydrolysis of inositol phospholipids (InsPL hydrolysis) produces "second messengers" that act as PKC activators or Ca^{2+}-mobilizing agents. In particular, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) may mobilize Ca^{2+} from intracellular or extracellular compartments, respectively. Other inositol phosphate (IsnP) isomers (including several positional isomers), whose synthetic pathways, metabolic fates and biological activities

are not clearly understood, are produced following receptor stimulation. Diacylglycerol (DAG), coproduced with the hydrolysis of any InsPL precursor, acts as an endogenous activator of PKC. In addition, hydrolysis of other cellular phospholipids (i.e.: phosphatidylcholine) may be another source of DAG.

In spite of the ability of PKC activators and Ca^{2+} mobilization to induce T cell activation, the functional role of the InsPL hydrolysis pathway is not clear. We have provided evidence dissociating the TCR-induced InsPL hydrolysis response from certain biological responses. Nonetheless, this metabolic parameter is an important tool for understanding signal transduction via the TCR.

The mechanism(s) of TCR coupling to the enzymes involved in InsPL hydrolysis, the relationship and "cross-talk" between this pathway and other signal transduction mechanisms (e.g.: PKA, PKC, tyrosine kinase and Ca^{2+}/calmodulin-regulated kinases), and the role of other signals (e.g.: hormonal receptor (prostaglandin)-mediated signals or adhesion molecules) in modulating InsPL hydrolysis will be addressed in this project.

What couples TCR/CD3 to PLC? A potential role for a guanine nucleotide-binding protein (G-protein) in CD3 coupling to PLC, based on the observation that activation of InsPL hydrolysis is induced by non-hydrolyzable guanine nucleotide analogs and is inhibited by a G-protein inhibitor, GDPβS. However, activation of protein tyrosine kinase activity(ies) is also associated with perturbation of the TCR/CD3 complex, with protein tyrosine phosphorylation of several cellular substrates including the ζ chain of the CD3 complex. Activation of protein tyrosine kinase activity(ies) may be required for phospholipase C activation, for the following reasons: protein tyrosine phosphorylation may preceed PLC activation; inhibitors of protein tyrosine activation inhibits PLC activation (note potential problems with direct effect on PLC activity); a PLC isozyme, PLC-γ1is phosphorylated on tyrosine residuces after CD3 ligation. A src famil kinase, fyn, is a potential candidate as a mediator of CD3-induced T cell responses, as indicated by : coprecipitation of p59fyn protein trosine kinase with the CD3 complex, and co-crosslinking of CD3 ζ-chain to p59fyn by DTSSP in permeabilized cells. A potential role in signal transductio has also been suggested for CD3 ϵ chain, which may be coupled to a protein tyrosine kinase (unknown identicty), as indicated by the pattern of protein tyrosine phosphrylation observed in of functionally active transfectancts expressing either CD3 ϵ chain or CD3 ζ chain.

What regulates the inositol phospholipid hydrolysis signal transduction pathway? The existence of an inhibitory cross-talk between the cAMP-dependent pathway and the PLC pathway is suggested by the observation that T cell activation is inhibited by cyclic nucleotides (cAMP), the inhibition correlates with blockade of CD3-induced PLC activation, and that cAMP-dependent protein kinase A (PKA) phosphorylates PLC and inhibits InsPL hydrolysis in permeabilized T cells. Furthermore, an inhibitory feed-back role of cytoskeletal elements exists, as indicated by the findings that TCR/CD3 perturbation induces actin polymerization, and that disruption of actin polymerization results in potentiation of TCR/CD3-induced PLC activation and Ca^{2+} mobilization. Intracellular Ca^{2+} levels may also play a role in he control of signalling. In fact, free Ca^{2+} concentrations of the order of magnitude that may be obtained intracellularly upon T cell ativation inhibit inositol phosphate generation and stimulate Ins(1,4,5)P_3 conversion to Ins(1,3,4,5)P_4, thus limiting the intracellular level of Ins(1,4,5)P_3.

In summary, these evidences suggest the existence of redundancy in both TCR/CD3 signaling pathways and coupling mechanisms. In other words, the TCR/CD3 complex is likely to be coupled to multiple signal transduction pathways (e.g., PLC, tyrosine kinase), each one per se may be sufficient for cell activation. Furhermore, coupling, such as that of PLC to TCR/CD3 may involve different mechanisms, and different CD3 chains may be coupled to different *src* kinases. In addition, at least two mechanisms may paly a feed-back role: actin polymerization and cAMP/PKA activation may control PLC activation

PREVIOUS RESULTS

A variety of murine T cell clones specific for the Ag, keyhole limpet hemocyanin (KLH) were generated and maintained in culture under two different growth conditions: lines maintained with Ag and

APC in addition to IL2 and lines maintained with IL2 alone (IL2-dependent lines). The MoAb, 2C11-145, directed against the ϵ chain of the CD3 reacted with all the clones and was used as universal stimulus. One of these clones expresses the epitope on the $V_{\beta 8}$-encoded determinant of the TCR recognized by the MoAb F23.1. Ag stimulation or perturbation of the TCR Ti or CD3 epitopes by the respective MoAb, induced T cell activation (measured by proliferation or release of specific granules) as well as rapid (within seconds) hydrolysis of cellular InsPL. We and others have in the past demonstrated activation of InsPL hydrolysis in human, Ag-specific, T cell lines in response to both Ag stimulation or MoAb (OKT3 MoAb) perturbation of the CD3 determinant.

The regulation of this pathway was further characterized in the convenient mouse T lymphocyte model. InsPL hydrolysis induced by 2C11 or F23.1 Ab depended on the presence of exogenous Ca^{2+}. Simply increasing the intracellular Ca^{2+} concentration by treatment with a Ca^{2+}-ionophore was insufficient to induce hydrolysis, suggesting the existence of a Ca^{2+}-dependent component of the activation signal proximal to the generation of the InsP.

A strategy based on permeabilization with bacterial lysins was used to characterize the mechanism of activation of PLC and its coupling to the TCR/CD3 complex. It was found that the non-hydrolysable guanine nucleotide analog, GTPγS, activates InsPL hydrolysis in permeabilized cells, suggesting that a guanine nucleotide-binding protein (G-protein) is controlling PLC activity.. The GDP analog, GDPβS inhibited InsPL hdrolysis induced by TCR/CD3 perturbation, indicating that a G-protein was likely to have a role in the modulation of InsPL hydrolysis.

Negative regulation of T cell activation was exerted by agents (e.g.: prostaglandins) known to mediate their action via activation of the cAMP transduction pathway. We have found that this effect of cAMP, together with the inhibitory effect mediated by other cell surface receptors, correlated with their ability to inhibit InsPL hydrolysis. This effect was mimicked by other agents known to activate the AC/cAMP/PKA pathway, but not by the PKC activator, TPA. The effect was observed in intact cells exclusively, but not in permeabilized cells even when millimolar amaounts of the nucleotide were used, suggesting that cytosolic mediator(s) may be involved in the inhibitory effect. PKA, which is mostly cytoplasmic, is a likely candidate. Reconstitution of permeabilized cells with the catalytic subunit of PKA resulted in inhibition of InsPL hydrolysis associated with the phosphorylation of specific membrane-associated proteins, including PLC-γ1. No phosphorylation of CD3 complex polypeptide by PKA was observed.

Cytochalasins, drugs that prevent actin filament polymerization and, therefore, block cytoskeleton assembly, increase the initial velocity and the maximal amount of InsPL hydrolysis triggered by perturbation of the TCR/CD3 complex obtained by a combination of an anti-CD3 Ab and a second Ab. Perturbation with immobilized (bead adsorbed) anti-CD3 Ab, per se resulting in a stronger signal than anti-CD3 plus a second Ab, is not further potentiated. The cytochalasin-mediated potentiation of CD3 signal did not correlate with the compartmentalization of CD3 between detergent soluble and insoluble fractions, a putative indication of receptor association with cytoskeletal elements. Rather, a decrease internalization rate was associated with the ability of cytochalasins to potentiate CD3-induce InsPL hydrolysis. This is not, however, the only component of the cytochalasin effect, since potentiation of CD3-induced InsPL hydrolysis by cytocahlasin treatment was observed even when prevention of receptor internalization was obtained by physical means (i.e., immobilized antibodies).

By the use of a novel HPLC method previously developed in our laboratory, the spectrum and metabolic fate of the various species of inositol phosphates produced upon CD3 perturbation were characterized. Metabolic stdudies as well as the effect of the intracellular free Ca^{2+} concentration on InsPL hydrolysis were performed employing permeabilized lymphocytes.

In collaboration with thelaboratory of Dr. L. Samelson, we have characterized the stoichiometry of CD3/fyn association by using permeabilized 2B4 hybridoma cells and chemical cross-linking. The results indicate that approximately 5% of cellular fyn kinase is associated with 25% of CD3 complexes. Preferential association with the CD3 ζ chain was laso observed.

In collaboration with Drs. G. VanSeventer and S. Shaw, we have investigated the role of ICAM-1/LFA-1 interaction in modulating CD3-insuced InsPL hydrolysis in human T lymphocytes.

Costimulation with immobilized anti-CD3 Ab and ICAM-1 induced prolonged (up to 4 h) InsPL hydrolysis, a condition that may lead to prolong signalling, an essential requirement for driving T cells to full activation.

OPEN QUESTIONS:

— Is $p59^{fyn}$ or any other kinase of the *src* family upregulated upon ligation of CD3? Protein tyrosine kinases of the src family are upregulated by dephosphorylation of a negative tyrosine phosphorylation site and autophosphorylation of a positive tyrosine phosphorylation site. While in other cells (e.g., B lymphocytes, RBL cells) ligand-induced activation of src-related kinases have been shown, no such evidence is still avalaible in T lymphocytes.

What phosphorylates PLC and how is PLC phosphorylated? Although it has been shown that PLC is phosphorylated on tyrosine residuces, no evidence of a responsible *src* kinase has been obtained in any cell. EGF or PDGF receptor kinases (an integral part of the cytoplasmic domains of these receptors) phosphorylate PLC-γ1 *in vitro* and *in vivo* and their interaction is mediated via *src*-homology region 2 (SH2) domains on both proteins.

Are negative feed-back mechanisms (cAMP, Ca^{2+}, microfilament assembly) affecting PLC functional activity or its coupling to TCR/CD3?

What is the functional result of knocking out PLC in T lymphocytes? Functional inactivation of PLC (different isozymes are prent in T cells: PLC-γ1, PLC-γ2, and PLC-B) should allow one to develop a model to study PLC-independent signal transduction mechanism and the role of individual PLC's in signalling (e.g., prolonged signalling).

MATERIALS and METHODS

Cells. Several murine, Th ($CD3^+$, $CD4^+$, $CD8^-$), Ag-specific, IL2 responsive, cell lines and clones are maintained in our laboratory. All have been characterized for expression of surface markers and showed a stable phenotype when maintained in culture for prolonged periods of times. Frozen master and working cell banks are available for re-expansion of the original clones. Clones are routinely maintained in culture with periodic stimulation by Ag-pulsed, irradiated feeder cells. Certain cell clones have been adapted to grow in culture with IL2 alone.

One clone has shown a decreased or absent cAMP response to forskolin (FSK), an inducer of AC activity. The basis of this defect is not defined and will be characterized in the context of this project.

Antibodies. MoAb directed against several cell surface markers are available in our laboratory. These include 145.2C11 (hamster MoAb anti-mouse CD3, ε chain), H57 (hamster anti-TCR), F23.1 (mouse MoAb, anti-mouse TI, $V_{\beta 8}$-encoded determinant of the TCR), GK1.5 (rat MoAb, anti-mouse CD4), and G7.4 (mouse MoAb, anti-mouse Thy-1.2 Ag). MoAb are affinity purified from mouse ascites fluid injected with the Ab-producing hybridoma clones. Papain-derived Fab' fragments have been obtained from the 145.2C11 MoAb.

Lipid biochemistry. The metabolism of membrane lipids and their products by T lymphocytes is studied using tracer-based methodology. All the relevant techniques have been developed, adapted to be used with radioactive materials, and validated by our laboratory. These techniques are based on the radioisotopic labeling of the precursor phospholipid pool in responder cells and the generation of labeled products upon cell activation. By using different metabolic precursor, individual pathways may be studied. All the assays were optimized for the utilization of tracer-based methodologies. Phospholipid and neutral (non-polar) lipid extractions and chromatographic analysis are currently performed according to standard techniques (chloroform / methanol / water partition, methanol /heptane partition, etc.). Quantification is based on scintillation counting and/or radioactivity scanning. Phospholipid and neutral lipid separation by several thin layer chromatography (TLC) systems (uni- and bi-dimensional) are currently established in our laboratory. Quantitation of mass amounts of phospholipids is performed as assay of inorganic phosphorous upon acid hydrolysis. Fatty acid composition of glycerolipids, if deemed necessary, may be obtained by gas chromatography separation of fatty acids methyl esters after methanolysis.

Inositol phospholipid hydrolysis assay. InsPL hydrolysis is measured as the amount of InsP produced from myo-[^{3}H]-inositol-labeled cells. InsP are separated by ionic exchange chromatography using AG1-X-8 resin in a rapid, single- (total InsP pool) or multiple-step (individual isomers) elution, depending on the type of isomer resolution required. Separation of phosphorous positional isomers of the InsP may be obtained by HPLC employing traditional anionic exchange chromatography. A new HPLC method employng a reversed phase column and a micellar mobile phase has been developed and its improved characteristics of sensitivity and resolution makes it ideal for the analysis of cell metabolites.

Cell fractionation. A method for cell fractionation into particulate and cytosol fraction has been established and validated to yeld high recovery of enzymatic activities from Th cells. High resolution fractionation of subcellular particles has been obtained by Percoll gradient centrifugation.

Diacylglycerol assay. This assay is based on the quantitation of the amount of ^{32}P incorporated into phosphatidic acid from [^{32}P]-ATP as a result of a diacylglycerol kinase-catalized reaction under excess of enzyme. The technique has been optimized and its specificity characterized in our laboratory.

Protein kinase C assay. PKC assay has been established in our laboratory as the evaluation of the amount of ^{32}P incorporated from ATP-γ[^{32}P] into type I histones by cell membrane or cytosolic extracts in the presence of Ca, phosphatidylserine and a DAG (diolein).

Phosphoinositide kinase assay. This assay measures the synthesis from PtdIns of the phosphorylated precursors (PtdInsP and $PtdInsP_2$) of the InsP. The enzyme (or enzymes) involved may be receptor regulated. The assay is based on the evaluation of the amount of ^{32}P transferred from ATP-γ[^{32}P] into PtdInsP or $PtdInsP_2$ in the presence of membrane preparations. Phospholipids are separated by TLC.

HPLC analysis of inositol phosphates. The laboratory has previously developed a novel technique based on a combination of micellar an dion-pairing chromatography. This techniques offers several advantage compared to standard ionic exchange chromatography, particularly with respect to sensitivity in radioactivity detection of low energy β particles.

Immunoprecipitation and immunokinase assay. Immunoprecipitation tecniques (anti-TCR/CD3 complex using the MoAb 145.2C11, anti-PLC, anti-phosphotyrosine, anti-lck, anti-fyn, etc.) have all been established in our laboratory and optimized for the precipitation of phosphorylated proteins. A variety of detergents have been used and the combination of digitonin and CHAPS was found to be optimal for CD3/TCR immunoprecipitation. Other Ab directed against other cell surface epitopes will also be used. Immunokinase assay is used to detect the functional status of protein kinases immunopurified with specific antibodies or associated with other cellular components.

Anti-phosphotyrosine Ab and related techniques. Ab recognizing phosphotyrosine (PY) residuces has been used in Western blot analysis of phosphoproteins. Other suitable Ab are also used for Western blot analysis. Non-radioactive, chemiluminescence-based technique has also been established for increased sensitivity, albeit lacking the possibility of accurate quantization.

Additional techniques. Bidimensional SDS-PAGE, protein column chromatography, analytical HPLC, etc. will be performed according to established methodologies. The laboratory has also capability for basic molecular biology techniques (DNA and RNA extraction and electrophoresis, Northern blot or dot-blot analysis).

GENERAL INVESTIGATIONAL PLAN

Three areas of research will be given priority for development:

1. Regulation of TCR/CD3 coupling to InsPL metabolism and hydrolysis, including cross-talk between TCR-induced InsPL hydrolysis and other second messenger-derived signals (i.e.: cAMP/PKA, PKC, Ca^{2+}/calmodulin) or tyrosine kinases.
2. Ca^{2+} regulation of InsPL hydrolysis and InsP metabolism.
3. Role of the cytoskeleton in TCR/Ag interaction and signal transduction.

COLLABORATIVE PROJECTS

1. Role of adhesion molecules in the activation signal via TCR/CD3 perturbation in human peripheral blood T lymphocytes.

RELEVANCE

The ability to design a pharmacological strategy aimed at the modulation of the immune cell function may only stem from an understanding of the molecular mechanisms controlling T lymphocyte activation. The current experimental immunosuppressant or antilymphoproliferative treatment with MoAb directed against TCR structure (i.e.: the mouse anti-human CD3 MoAb, Ortho OKT3) is an example of implementation of a therapeutic strategy whose mechanism of action, not yet entirely defined, may also reside in the ability of the drug to activate the metabolic pathways described above.

Other benefits may stem from our approach. These may include the generation of agents (such as MoAb) directed at receptors other than the TCR, their coupling elements or their effector molecules (i.e.: PLC) which may play a role as immunomodulators.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH-02026-01-LCB

PERIOD COVERED

October 1, 1991 through September 1992

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

Development of Xenogeneic antibodies to murine splenic lymphocytes

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

Vijaya Manohar, Ph.D., Senior Investigator
Sylvia Henry, Biologist
John Jessop, Ph.D., Senior Investigator
Thomas Hoffman, M.D., Chief
Thomas Chused, M.D., Medical Officer
Elinor Brown, Microbiologist

COOPERATING UNITS (if any)

Laboratory of Immunology, NIAID/NIH
Laboratory of Developmental and Molecular Immunity, Molecular Genetics of Immunity Section, NICH/NIH

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Division of Hematology

INSTITUTE AND LOCATION

Center for Biologics Evaluation and Research

TOTAL STAFF 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.)

The overall goal of this project is to develop exnogeneic monoclonal antibody reagents recognizing unique surface antigenic receptors on murine lymphocytes and their precursors. Female Wistar rates were immunized with single cell preparations of whole spleen from New Zealand Black Mice. Hybridoma cell lines were generated by fusing immune rat spleen cells with non secreting NS-1, myeloma cell line. A hybridoma clone VMM-2 was found to secrete an $lgG2_b$ antibody recognizing antigenic receptors on cells from bone marrow, spleen but not from thymus by flow microfluorometry. VMM-2 binds specifically to IgM^+ B cells, but the antigen recognized does not relate to any lineage specificity. Further, the antibody was found to be specific for the cells of the $H-2^d$ haplotype. Genetic mapping using a panel of recombinant inbred and congenic mice, revealed that the antigenic site is located at the K end of the MHC complex. Further studies, using a series of transfected cell lines expressing chimeric $H-2K^d$ gene products on their membranes has confirmed that the antigenic determinant is the region between the residues D152 to S184, located at the C terminal end of the a2 domain of the K^d molecule. A comparative Western analyses of lysates from T, B and monocyte cell lines ($H-2^d$) has indicated that in addition to binding to the monomeric form (45000 m.w), of the class I molecules from all the cell types (similar to a conventional anti-K^d), Vmm-2 specifically binds to a protein of approximately 180-200,000 m.w. Experiments are in progress to further determine whether the high molecular weight protein recognized by VMM-2 is really the tetrameric form of the class I molecule.

Objectives: To develop xenogeneic monoclonal antibody reagents recognizing unique surface antigenic receptors on the splenic lymphocytes and their precursors of New Zealand Black mice.

Methods:

Female Lewis rats were injected i.p. with 10^7 NZB spleen cells mice three times at weekly intervals. Three after the third injection, the rat spleen cells were fused with exponentially growing 8-azaguanine-treated, nonsecreting P3NS-1 myeloma cells in the presence of 35% polyethylene glycol. The fusion mixture at a cell density of 2 X 10^5 cells/ well were initially cultured in the HAT medium, to select hybrid colonies. The supernatants from the wells containing hybrid cells were screened for the antibodies binding to NZB spleen cells, by flowmicrofluorometry (FMF). Hybrid cells from positive wells were cloned by limiting dilution on irradiated BALB/c thymocytes as feeder layer.

Culture supernatants from positive clones were purified by DEAE column chromatography followed by gel filtration. Purified antibody was typed to be of a rat $IgG2_b$ antibody by using a monoclonal isotype typing kit and conjugated with fluorescein isothiocyanate by standard methods .

The tissue distribution of the antigen recognized by VMM-2 was determined by testing the cells from marrow, lymphnodes, spleen and thymus. The relationship of VMM-2 determinant to other cell surface antigens was tested by a series of blocking experiments, wherein, the percentage blocking of the antigenic site by various antibodies was calculated. To determine whether the putative antigen is an activation antigen, spleen cells from BALB/c mice stimulated with LPS were employed. To determine whether glycosylation of the antigenic determinant would affect the nature of VMM-2 binding, the target cells were either endogenously treated with tunicamycin or in vitro treated with endoglycosidase . To determine whether, VMM-2 is recognizing a circulating or cytoplasmic antigen, either target cells were either blocked by NZB serum and stained by VMM-2 or the cells fixed by methanol were stained by FITC labelled FITC.

A panel of recombinant inbred and congenic mice with various haplotypes, a series of transfected cell lines expressing chimeric H-2 K^d gene products and mouse anti-mouse, and rat anti-mouse H-2, and other lineage sp ecific hybridoma antibodies were employed to determine the serological specificity of the hybridoma antibody (VMM-2), by FMF. Antibody reagents were either directly fluoresceinated or counter stained with a fluoresceinted Fab_2 portions of the appropriate second step antibody. One million cells from spleen, thymus , bone marrow or cultured cell lines were reacted with either directly labelled or culture supernatants containing antibodies followed by labelled second step antibody and tested for specific immunofluorescence by FMF.

To detect the VMM-2 binding antigen, lysates of T, B, and monocyte cell lines of H-2^d haplotype were examined by Western analyses. Lysates were run either on a 8 or 14% PAGE-SDS (either reducing with 2-ME or non-reducing), either directly stained with coumassie blue or transferred to Immobilon-P membrane, stained with the conventional anti-K^d and VMM-2 antibody followed by second step antibody coupled to alkaline phosphatase (ELISAmate staining kit from KPL).

Results:

A hybridoma cell line secreting rat anti-mouse monoclonal antibody of $IgG2_b$ class was established. The tissue distribution by FMF analyses, showed that VMM-2 selectively stained cell populations from bone marrow and spleen while cells from lymphnodes and thymus were undetectably (dull) stained. the positive population in the spleen was further identified to be IgM^+ B cells. Where as T cells were not detectably stained. Further, the fluorescence intensity of the stained population was moderate.

Experiments to determine, whether VMM-2 is binding to an activation antigen, or circulating antigen or a cytoplasmic antigen was ruled out by unchanged binding intensity (by FMF). Additionally, moderate staining by VMM-2 was not due to weak binding affinity, as increased concentrations of the antibody or prolonged incubation of the stained cells did not affect the fluorescence intensity. Further, the treatment of antigenic determinant by anti-glycosylating agents did not affect the binding intensity, suggesting VMM-2 may be specifically binding to a fine epitope.

The strain distribution of VMM-2 binding using a panel of recombinant inbred and congenic mice revealed that, VMM-2 is specifically binding to the cells from H-2 haplotype. In that, the cells from mice specifically carrying the K end the 'd' haplotype were found to be stained by VMM-2. Blocking experiments have further confirmed that VMM-2 specifically binds to a receptor on spleen cells. These results suggested that VMM-2 may be either binding to a unique H-2 antigen located at the K end of the MHC complex or recognizing a polymorphic form of class I gene product preferentially expressed on B cells of H-2^d mice. Further, this antibody did not cross react with any other

haplotypes. Analyses of transfected L cells expressing chimeric H-2 K^d gene products showed that VMM-2 binds to the cells expressing epitopes corresponding to the residues D152- S184 , located at the C terminal end of the α2 domain of the K^d molecule. These results clearly prove that VMM-2 binds to a fine epitope which is located at the bottom part of the molecule.

Immunoblot analyses of lysates from lineage specific cell lines of H-2^d haplotype showed that VMM-2 and a conventional anti-K^d antibody bind with similar intensity to a 45000 m.w. protein on all the cell types tested, in the order of B cells > T cells>I monocytes. Additionally, VMM-2 was found to stain a protein or proteins approximately of 200,000 m.w.

Significance:

VMM-2 is an unique anti-H-2 K^d antibody recognizing one or two of the antigenic epitopes (out of 3 known)of described to be located on the α2 domain of the class I molecule, where T cell receptor of CD-8^+ cells is believed to bind. So far no antibodies recognizing this part of the molecule has not been reported. Hence VMM-2 could be a valuable tool for the studies on interaction between class I and $CD8^+$ T cells.

VMM- 2 uniquely recognizes a high molecular weight protein on B cells, which is not recognized by a conventional anti-K^d antibody. Recent reports show that class I molecules being polymorphic can occur as dimeric or tetrameric forms on T cells under certain physiological conditions such as viral infection or endogenous peptide processing. In the light of this evidence, it is conceivable, that high molecular weight protein recognized by VMM-2 on B cells may be those oligomeric cluster of class I molecules. As B cells are not involved in alloreactivity, the latter hypothesis may be applicable. Then, VMM-2 could be a valuable tool in dissecting the T-B interaction in apoptosis.

Proposed course:

Experiments are under way to further show that the high molecular weight protein stained by VMM-2 is really oligomeric molecule. In that, blots will be repeated to show that on a reducing gel polymeric forms will dissociate into monomers and all the binding by VMM-2 is at 45000 m.w. Additionally, the high molecular weight protein will be extracted, treated with reducing agents and detergents to gently separate monomers and show that VMM-2 still specifically binds to monomer, and the intensity staining on the western blot is due to the conglomeration of the monomers.

Upon completion of the above experiments it is anticipated to submit the manuscript for publication.

Laboratory of Cellular Hematology

Annual Scientific Report - 1992

— The Laboratory of Cellular Hematology (LCH) has continued to make progress in studies of leukocytes, platelets and hemoglobin-based blood substitutes.

A. Specific projects:

I. Platelets.

A. Studies related to platelets for transfusion.

The demand for platelet transfusions continues to increase, despite the decrease in demand for red cells and recent studies indicate that these trends are likely to persist. At the NIH-sponsored meeting (April 1991), Cellular and Molecular Basis of the Platelet Storage Lesion, there was a consensus that neither the platelet product nor assay methods for platelets are satisfactory. We will continue to seek and study approaches to development of practical assay of platelets for transfusion. Dr. Prodouz has served as the lead investigator on this project, until her departure in July, when that position was assumed by Dr. Jaroslav Vostal. Dr. Fratantoni devotes time to the project and they are aided by Dr. Pastakia, Ms. Keville and Ms. Poindexter.

1. We continue work on a program to seek and study approaches to development of practical assay methods for platelets for transfusion. We have available several very sensitive methods for identifying storage-induced changes in the platelet membrane, membrane skeleton and cytoskeletal proteins. In collaboration with Douglas Terle, we are using flow cytometry to investigate the behavior of cellular components that reflect storage-induce activation.

We previously adapted microplate technology to measurement of platelet aggregation and have now adapted this technology to performance of platelet response to hypotonic stress. The technique permits use of hypotonic stress assays, which are widely used in platelet storage work, on a scale not previously possible. We are using this application on studies of the mechanism of the hypotonic stress reaction, as well as on investigation into other apsects of platelet reactivity.

2. We are studying various aspects of platelet storage with the goal of identifying means to improve product quality. We have begun studies of stimulus-response signal transduction, especially that related to transmembrane calcium flux. Since the elusive platelet "storage lesion"

exerts a pervasive effect on cell function, it is likely to be related to membrane effects, which would be reflected in signal transduction changes.

3. We have investigated the generation of microvesicles (MV) known to occur during storage of platelets and found that it involves calpain-induced proteolysis of cytoskeletal proteins, including actin. The results obtained thus far suggest that calpain activation occurs in platelets during storage and that activation increases over time. Further, preliminary results suggest that shear stress may contribute to microvesicle generation. Cytoskeleton disruption by cytochalasin D or vinblastine was not sufficient to cause microvesicle release or calpain activation.

4. Beta lactam antibiotics, such as penicillin, exert inhibitory effects on platelets, in part, by binding to the platelet surface and interfering with agonist-receptor interactions. We have studied this system as a possible probe for understanding platelet activation and have found that penicillin G induces platelet dysfunction by impairing the activation -linked regulation of surface receptors. We are extending the experience obtained with this project to an invistigation of the effect of amphotericin on the structure and function of the platelet membrane.

B. Studies related to viral inactivation of cellular components. LCC published the first report of significant viral inactivation by UV light under conditions that caused minimal damage to platelets. We have completed a number of studies designed to probe for mechanisms of inactivation methods and to seek approaches to the problems that are being presented as regulatory issues. We believe that further work in this area will concentrate on application of the principles already developed to commercial use. We completed our efforts in this area with a study of the changes in platelet reactivity related to viral inactivation procedures, which was a collaborative arrangement with CDRH.

1. Drs. Fratantoni and Prodouz were invited to give major presentations and contribute a textbook chapter on this subject.

2. We have investigated the antiviral activity of the photoactivatable compound gilvocarcin, an antitumor agent which is closely related to psoralen. It has been shown to be toxic to bacteria and mammalian cells at picomolar levels in the presence of UVA. We found that this agent did not inactivate two model viruses, ΦX174 and PRD1, and concluded

that gilvocarcin is not appropriate to photoinactivate viruses in blood products.

II. Leukocytes. We continue to study the mechanisms by which neutrophils migrate toward chemical attractants (chemoattractants). Leukocyte chemotaxis is a critical host defense mechanism involved in inflammatory responses to infectious, immunologic and neoplastic stimuli. The purpose of this program is to better understand the basic biochemical, physiological and structural requirements for leukocyte migration. Several approaches, including biochemical assays of cytoskeletal F-actin, fluorescent probe studies with flow cytometry and laser scanning confocal microscopy, and physiological studies directly measuring leukocyte migration, are used to dissect the mechanisms of leukocyte chemotaxis.

This year a series of CD45 negative RAW 264 macrophage mutant clones were generated and found to have defective chemotaxis. These clones will provide a useful set of tools to explore the molecular basis of chemotaxis. A project which has systematically evaluated the adhesion molecule expression on adherent and nonadherent human neutrophils exposed to chemoattractant, has provided new information on the down-regulation of adhesion molecules which is associated with chemotactic responsiveness.

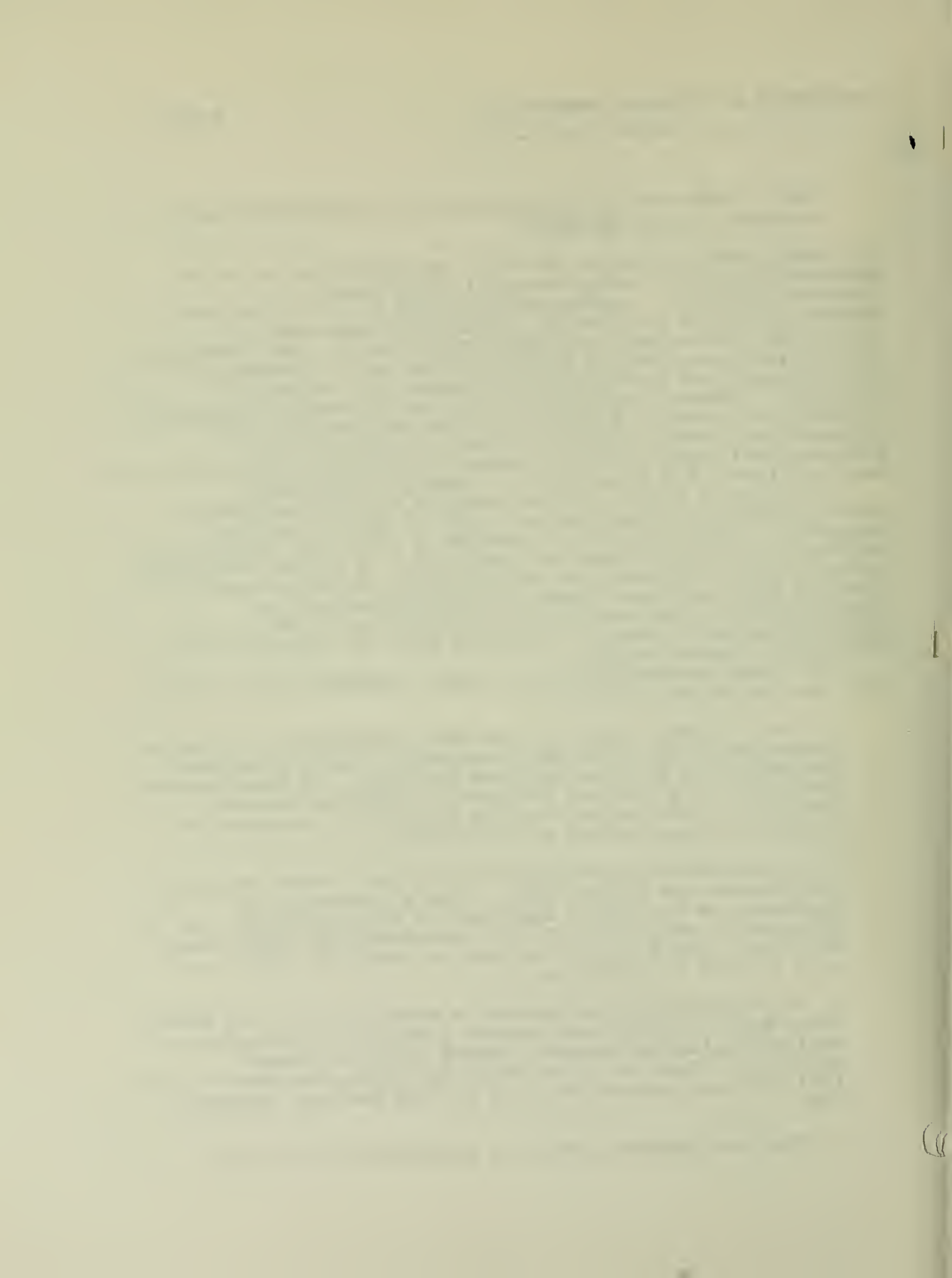
Dr. Liana Harvath leads this group, assisted by Mr. Douglas Terle and Ms. Nicole Brownson.

1. We initiated a study of the down regulation of adhesion molecules during neutrophil chemotaxis, using monoclonal antibodies to an array of these proteins. We have observed that there are distinct patterns of adhesion molecule expression correlating with the chemotactic responses of distinct subpopulations of neutrophils.

2. We successfully generated myeloid cell clones that did not express the antigen CD45. This glycoprotein is expressed on the plasma membranes of all leukocytes, but its function is not known. We are characterizing these mutant cells and intend to utilize them to probe the functional role of CD45 in myeloid cells.

3. We continued the collaborative study with Dr. Amy Skubitz (Univ Minn) to determine whether peptides corresponding to various regions of laminin induced human neutrophil migration. Results of the study have been presented at the 1991 American Society for Cell Biology Meeting, December 1991.

4. The flow cytometry facility continued to serve as a



resource for basic and clinical studies:

a) A collaborative study with Kristina Prodouz and Katie Pastakia evaluated the surface expression of glycoproteins associated with exposure to penicillin. Publication in 1992.

b) Effects of nerve growth factor and gamma interferon on the subpopulations of a neuroblastoma cell line. Presented at World Congress on Cell and Tissue Culture, 1992.

c) Characterization of adhesion molecule expression during chemotzxis. Presented at AFIP Conference on Quantitative Histopathology, 1992.

5. A laser scanning confocal microscopy system was set up in 1989. This is a state of the art technology used in several studies to obtain detailed morphologic information on cytoskeletal architecture, membrane antigen location and intracellular distribution of organelles. Current projects:

a) Role of neutrophil cytoskeleton and integrin molecules on adherence strength.

b) Myeloid membrane antigen location during leukocyte migration.

III. Hemoglobin - Blood Substitutes. In response to the increasing regulatory activity related to red cell substitutes, LCH established this new program on 1989. Most of the red cell substitutes under investigation are modified hemoglobin solutions, and it was felt that a laboratory and personnel with interest and expertise in hemoglobin chemistry would be appropriate. Dr. Abdu Alayash has lead responsibility for this scientific program, assisted by Ms Beth Brockner Ryan.

The following research projects are underway:

1. Modified hemoglobins as a source of activated oxygen species. We have established that chemical modification used to generate hemoglobins cross-linked at either the or ß chains alter their ability to generate or interact with oxygen free radicals. This form of oxidative modification may emerge as a physiologically important event which may lead to a significant contribution to reperfusion injury.

2) The formation of altered heme products by human hemoglobins. We have asked the question of whether chemically modified hemoglobins developed as blood substitutes undergo similar structural and/or enzymatic modifications which may explain, at least in part, some of the toxicities associated with the infusion of hemoglobin-based products such as vasospasm and reoxygenation injury. Results so far indicate that treatment of

hemoglobin with low levels of hydrogen peroxide produced soluble protein-bound heme products that are chromatographically similar to that of myoglobin. Work is under way to test for enzymatic activity in a variety of physiologically relevant reducing systems.

3) Nitric oxide binding to cross-linked human ferrihemoglobins. We have explored the interactions between NO and oxidized human hemoglobin, comparing the behavior of unmodified HbA_0 with that of two chemically cross-linked hemoglobins. The rates of binding, and rates of reduction from Fe^{III}-NO to Fe^{II}-NO were altered in the modified hemoglobins, indicating that cross-linking changes the alpha and beta chain heme pockets. If the vasoconstrictive effects of extracellular hemoglobin involve cycling between oxidized and reduced forms in response to exposure to NO, the differences in rate constants observed for the modified hemoglobins may prove to be of physiological significance.

4) Interactions of modified hemoglobins with iron chelators: desferrioxamine and the pyrone ethyl maltol. Among the current strategies aimed at decreasing the oxidant effect of HBOCs is the use of iron chelators to prevent the potential molecular and cellular damage caused by iron induced oxygen free radicals. We found that there was a slight decrease in both the rate of autoxidation and hemichrome formation when ethyl maltol was included in the solutions containing these hemoglobins. Conversely, inclusion of desferrioxamine led to an increase in oxidation products.

5) Effects of hypothermic conditions on the oxygen carrying capacity of crosslinked hemoglobins. In view of the potential application of HBOCs in perfusion of organs for cardioplegia during major surgery, or for preservation of donated organs at low temperatures before transplant, we examined temperature dependence profiles of oxygen equilibrium curves at 15-37° C for a number of human and bovine hemoglobins crosslinked with various diaspirin derivatives. We have found differences in the thermal effects oxygen equlibrium curves that correlate with structural modifications. These must be studied further if the products are to be used at varying temperatures.

B. Future Plans:

1. Platelets.- A. ~~related to platelet storage.~~ There is no substitute for platelet transfusion on the horizon. Thus, although red cell usage is decreasing and should decrease further, platelet usage is likely to increase. The available platelet product is of variable quality and current testing

methods for platelets are not satisfactory. Improved product and assay methodology will be needed if we are to limit the donor exposure of each patient receiving platelets. We will explore methods for better validating, with cell biology methods available to us, the optical technique developed by LCC several years ago so that it may be used for quality control - studies on membrane function and signal transduction will be paramount. We will seek other assay methods, using state of the art cell biology techniques, and evaluate them as potential QC methods - studies using flow cytometry and cell imaging will be attempted. Collaborative efforts will begin aimed at elucidating the role of calpain in protein changes seen during storage.

In 1981, we issued Guidelines for Platelet Testing that called for a three-phase test procedure: laboratory testing, autologous recovery and clinical testing. These expedited review and approval of new platelet container that permitted longer storage and improved quality. These Guidelines will be revised and the laboratory aspects modified to reflect the new assay methodology.

B. Related to viral inactivation. The studies have been completed and the results published or in preparation. The personnel involved, whose expertise is in the area of platelet physiology, will work on the platelet storage and assay problems. Collaborative projects with major blood centers involved with various viral inactivation modalities will be finalized.

Drs. Fratantoni and Prodouz were invited to give major presentations on this subject, produced an editorial published in TRANSFUSION and have prepared a chapter for a forthcoming major textbook.

2. Leukocytes.

Future Directions:

The goal of the leukocyte research program is to better understand the signal transduction mechanisms of leukocyte chemotaxis and activation. The CD45 molecule appears to play an important role in signal transduction for phagocyte motility and Fc receptor-mediated endocytosis. We will utilize the CD45 negative macrophage clones to explore the molecular basis of CD45 function in chemotaxis, phagocytosis, adherence, and microbicidal activity. A major effort will be spent on identifying the molecular defects in the CD45 negative clones and linking the defects with functional parameters of the cell. The leukocyte laboratory of LCH has been invited to participate in the Vth International Workshop on Leukocyte Differentiation Antigens. More than 300 monoclonal antibodies will be evaluated for their binding properties and effects on human neutrophil migration.

These studies will be part an international cooperative effort to better understand the functional role of myeloid membrane proteins.

3. Hemoglobin. We will continue studies on newly developed hemoglobins that are being studied as potential blood substitutes. We will emphasize studies on binding with nitric oxide and relationship of structural modification to oxidative stability. The role of hemoglobins in initiation and propagation of oxygen radical-mediated reactions will be studied using various modifications of hemoglobin. We will attempt to initiate collaborative arrangements to find an appropriate animal model to correlate the laboratory values with in vivo studies.

We will continue the collaborative efforts with one or more laboratories to provide us with the needed hemoglobin preparations to be used for the types of studies described.

Other Activities of the Staff.

Scientific review work within LCC continued to be quite active this year. The Laboratory will have reviewed some 400 IND, NDA and 510(k) submissions in 1992. In addition, there have been over 100 license applications and amendments dealing with platelets and plasma. Dr. Harvath was appointed chair of a major PLA committee.

Dr. Fratantoni was a member of the Blood Substitutes Panel of the Naval Research Advisory Committee. This group was mandated by Congress to evaluate the status of blood substitutes and report on the findings.

In 1992, we organized a Working Group on Viral Inactivation of Cellular Blood Products. This group, which involved staff from several locations within CBER, addressed the question of infectivity of blood products that have been treated to decrease microbial titer. The output from the group should permit CBER to provide answers to regulated industry.

All inquiries regarding blood substitutes, plastic containers and erythropoietin are referred to this Laboratory. These, along with an array of other issues that change with time, resulted in many hours of meetings and phone conversations devoted to technical discussion and consultation.

LCH Bibliography - 1992

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Vostal JG and Fratantoni JC, cAMP, but not econazole, inhibits store-generated signal in platelet store-regulated influx induced by thapsigargin. Biochem J., submitted

SELECTED PRESENTATIONS

Dr. Harvath:

"Differential Activation of Human Neutrophils with Oxidized N-formyl Peptides" presented to The Armed Forces Radiobiology Research Institute, Bethesda, MD., October 1991

"Laminin Peptides Stimulate Human Neutrophil Chemokinesis" at the Annual Meeting of the American Society of Cell Biology, Boston, MA., December 1991

"Flow Cytometric and Confocal Laser Scanning Microscopic Assays of Phagocyte Function" presented to The Institute for Advanced Training in Cellular and Molecular Biology, The Catholic University of America, Washington, D.C., May 1992

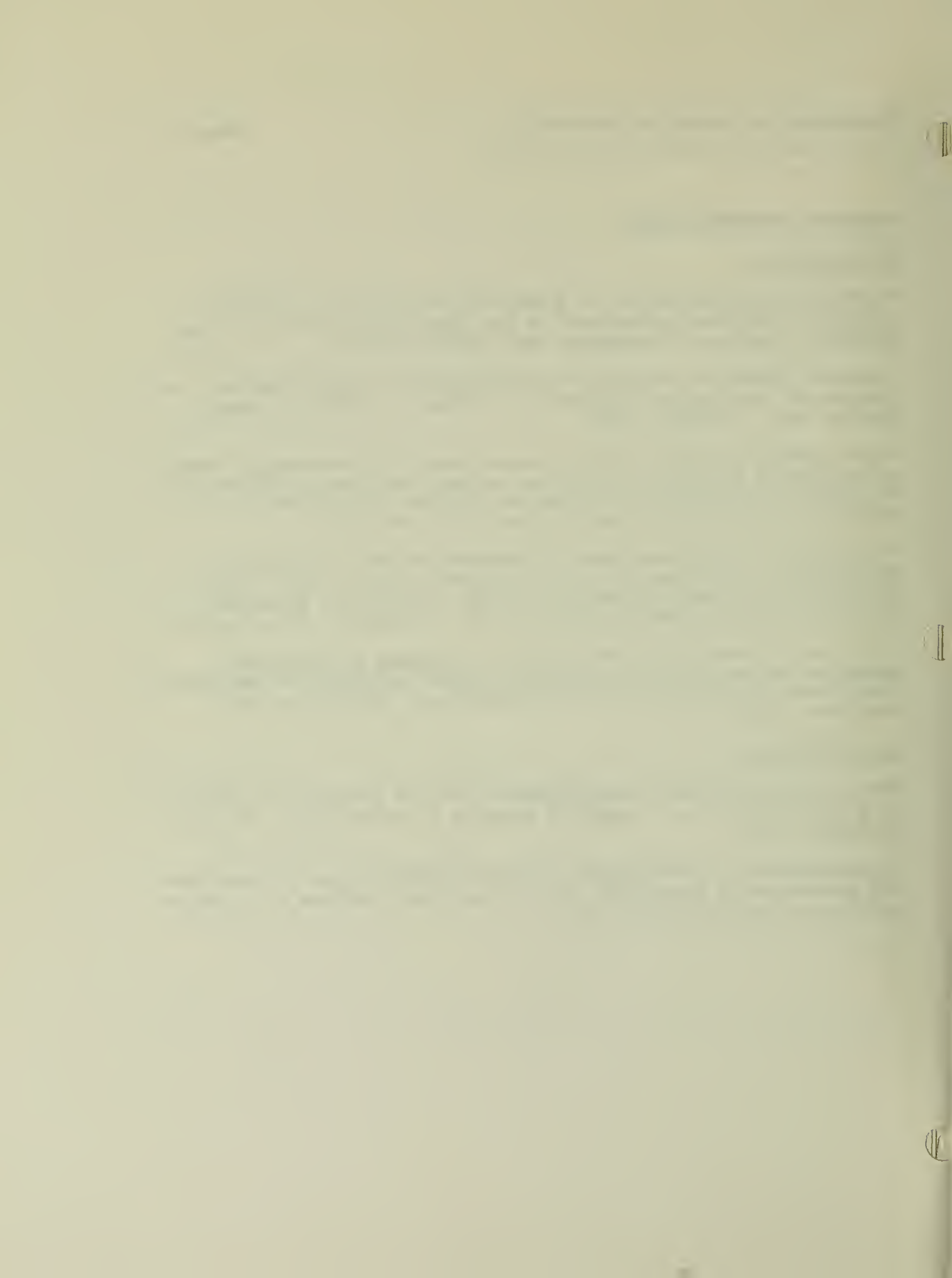
"Biphasic F-actin Responses in Chemoattractant Stimulated Neutrophils: Studies with Flow Cytometry and Confocal Laser Scanning Microscopy" presented to the Biological Response Modifiers Program Research Group, NCI, Frederick, MD., May 1992

"Confocal Microscopy of Chemotaxis: Changes in Neutrophil Adhesion Molecules" for the Armed Forces Institute of Pathology Conference on Quantitative Histopathology, Rockville, MD., September 1992.

Dr. Fratantoni

"Perfluorocarbon Blood Substitutes", at the Educational Session on Blood Substitutes, American Society of Hematology, Denver, December 1991.

"Alternatives to Transfusion, the New Technologies", (organizer and presenter) a symposium for the American College of Clinical Pathology, Boston, April 1992.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01030-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

ROLE OF GLYCOPROTEINS IIB/IIIA IN STORE-REGULATED CALCIUM INFLUX

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

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Staff Fellow
DH, CBER

Kristina Prodouz, Ph.D.
Research Chemist

Elizabeth Keville
Chemist

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular Hematology

SECTION

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0.6	0.6	

CHECK APPROPRIATE BOX(ES)

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

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

Glycoprotein IIb/IIIa complex mediates platelet aggregation by its ability to bind fibrinogen and has been implicated in regulation of platelet calcium influx. Platelets from individuals affected with Glanzmanns thrombasthenia lack the IIb/IIIa complex on their platelets and have decreased influx of calcium. Blocking of the IIb/IIIa complex on normal platelets with monoclonal antibodies or disrupting the complex by incubation with EDTA at 37 C also produces a decrease in calcium influx. Moreover, isolated IIb/IIIA complexes can act as calcium ion chanels when they are reconstituted into lipid vesicles. We have studied the mechanism behind store-regulated calcium influx in platelets. Calcium stores appear to have the ability to increase plasma membrane permeability to calcium when these stores are depleted of their calcium. This initiates calcium influx into the cell which is stopped when stores are refilled. The regulatory mechanism behind this influx is not known. We have investigated whether IIb/IIIa complex mediates the store-regulated calcium influx across plasma membrane. The glycoprotein IIb/IIIa complex was disrupted by treatment with EDTA and warming at 37 C as previously reported or blocked with the peptide RGDS which prevents fibrinogen binding to IIb/IIIa. Such treaments did not have an effect on thapsigargin-induced calcium influx. Thapsigargin can deplete intracellular stores and mimick store-regulated calcium influx. Thus IIb/IIIa does not appear to be the calcium channel operated by internal calcium stores.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01031-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

EFFECTS OF CAMP AND ECONAZOLE ON PLATELET CALCIUM INFLUX

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

Jaroslav Vostal, M.D., Ph.D.
Staff Fellow
DH, CBER

Joseph C. Fratantoni, M.D.
Laboratory Chief

Betty Poindexter
Biologist

COOPERATING UNITS (if any)

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0.6	0.6	

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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.)

Calcium in intracellular stores can control plasma membrane calcium permeability by an unknown mechanism. The imidazole anti-fungal agent, econazole has been proposed to inhibit store-regulated calcium influx in platelets by blocking the signal between empty internal calcium stores and the plasma membrane. We have previously suggested that such a signal may be related to calcium dependent tyrosine phosphorylation. To study the correlation between store-regulated calcium influx and tyrosine phosphorylation we utilized thapsigargin to deplete calcium stores and observed the effects of econazole and elevation of cAMP, an inhibitor of platelet tyrosine phosphorylation. Both econazole and cAMP inhibited thapsigargin-induced tyrosine phosphorylation and thapsigargin-induced calcium influx as measured by $^{45}Ca^{2+}$. Only cAMP decreased thapsigargin-induced efflux from $^{45}Ca^{2+}$ loaded platelets while neither econazole or cAMP elevation affected calcium efflux from resting platelets. When calcium stores were depleted with thapsigargin prior to addition of inhibitors so that the store signal to the plasma membrane was already generated only econazole continued to inhibit influx of calcium. These observations indicate that cAMP inhibits generation of the thapsigargin-induced store signal while econazole acts as a calcium channel blocker.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01032-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

ROLE OF VINCULIN IN PLATELET SIGNAL TRANSDUCTION

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

Jaroslav Vostal, M.D., Ph.D.
Staff Fellow
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0.4	0.4	

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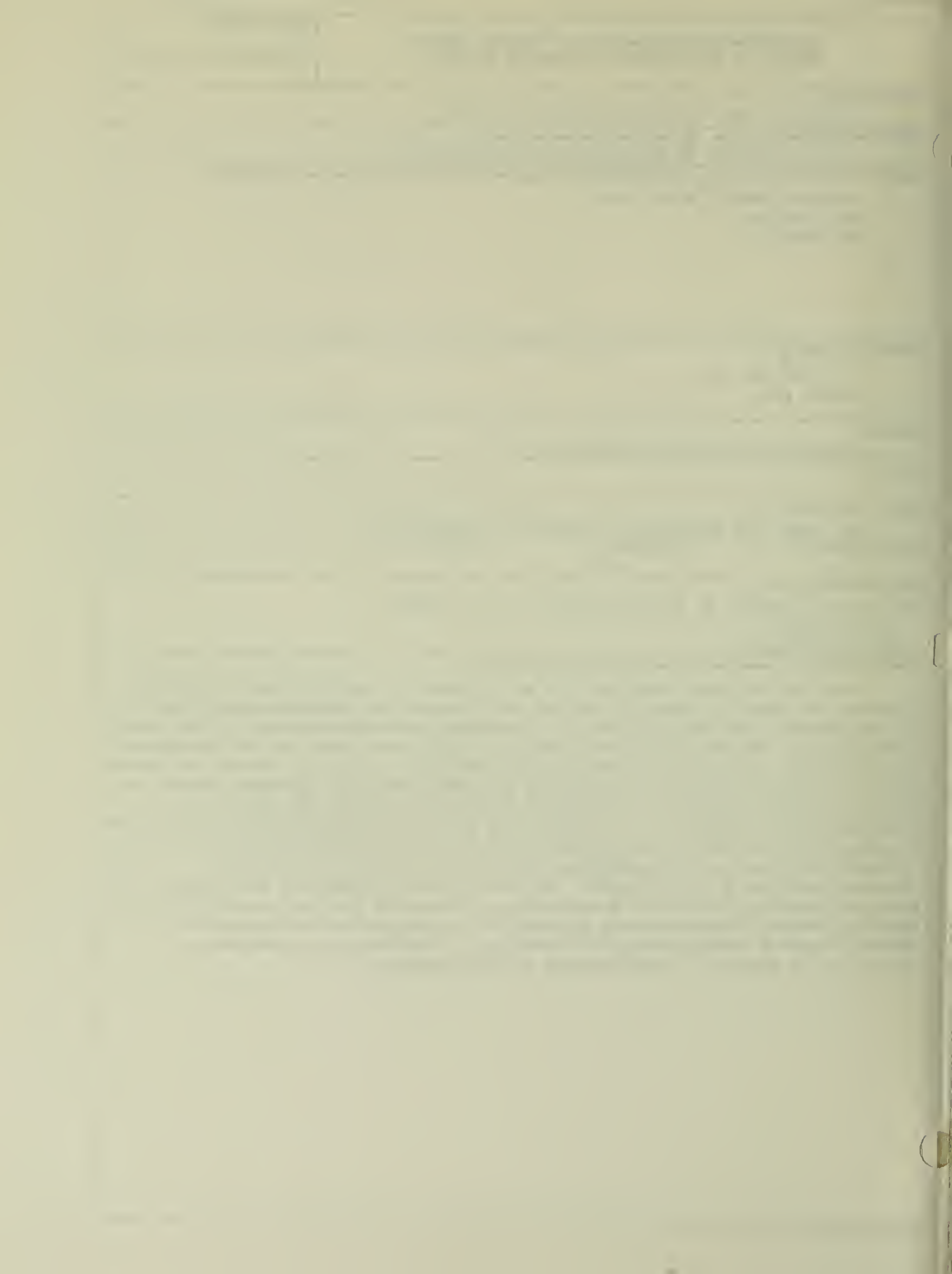
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

When intracellular calcium pools are released by agonists such as thrombin, elevation of platelet cytosolic calcium (Ca_i) induces tyrosine phosphorylation of a 130 kDa protein; and refilling the pools mediates dephosphorylation of this protein (Vostal et al. JBC 266:16911-16916,1991). In the present work the 130 kDa protein was identified as vinculin by the following criteria: 1) It is detected on western blots of thrombin-activated platelets by both monoclonal anti-phosphotyrosine and anti-vinculin antibodies. 2) It associates with the platelet Triton-soluble fraction as does vinculin. 3) It reacts with monoclonal anti-vinculin when isolated by affinity chromatography on agarose linked to anti-phosphotyrosine antibody. 4) It reacts with both anti-phosphotyrosine and anti-vinculin when focused isoelectrically at a pI of 5.4-5.8. 5) Its proteolytic fragments are reactive with both anti-phosphotyrosine and anti-vinculin. Since the level of stored calcium is known to control plasma membrane permeability to calcium and also appears to control tyrosine phosphorylation of vinculin, vinculin may be involved in regulation of platelet plasma membrane calcium channels.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01029-02-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Laminin Peptides Stimulate Human Neutrophil Chemokinesis

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

Liana Harvath, Ph.D.
Supervisory Microbiologist
LCH, DH, CBER

Nicole E. Brownson, B.A.
Biologist
LCH, DH, CBER

COOPERATING UNITS (if any)

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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.)

Laminin, an 850 kDa basement membrane glycoprotein, has been reported to stimulate directed cell migration (chemotaxis) of rabbit peritoneal exudate neutrophils (PMN). Human PMN express receptors for laminin, however, the chemoattractant activity of laminin for human PMN remains undefined. Laminin, isolated from Engelbreth-Holm-Swarm tumor, and ten synthetic peptides corresponding to various regions of the laminin A and B1 chains were compared for their abilities to stimulate human PMN chemotaxis and activated random migration (chemokinesis) through polycarbonate membrane filters in a 48-well microchemotaxis assay. Peptides F-9, F-11, F-12, and F-13 were derived from the laminin B1 chain cross-region (J. Cell Biol. 1988, 107: 1253), while six peptides were derived from the laminin A chain (J. Invest. Derm., 1991, 97: 141): peptide TG-1 from the amino terminus top globule; peptides GD-1, GD-3, GD-6, and GD-7 from the carboxy terminus globular domain; and peptide AG-1 from above the carboxy terminus globular domain. Laminin and the peptides were evaluated over a concentration range of 1-200 ug/mL in the motility assays. Only four of the peptides, F-12, TG-1, GD-6 and GD-1, stimulated PMN chemokinetic migration. Laminin and the other peptides failed to stimulate human PMN migration. In contrast, laminin stimulated rabbit peripheral blood PMN chemokinesis. These results demonstrate a species difference in human and rabbit PMN motility responses to laminin. Three peptides from the laminin A chain, TG-1, GD-6, and GD-1, and one peptide from the B1 chain, F-12, stimulate human PMN chemokinetic migration. These results suggest that specific laminin fragments which may be generated during basement membrane degradation can stimulate human neutrophil migration.

The results of this study were presented at the 1991 American Society for Cell Biology Meeting and are submitted for publication as a manuscript.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01033-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Down-Regulation of Adhesion Molecules During Neutrophil Chemotaxis

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

Liana Harvath, Ph.D. Supervisory Microbiologist LCH, DH, CBER

Nicole E. Brownson, B.A. Biologist LCH, DH, CBER

Douglas A. Terle, M.S. Biologist LCH, DH, CBER

COOPERATING UNITS (if any)

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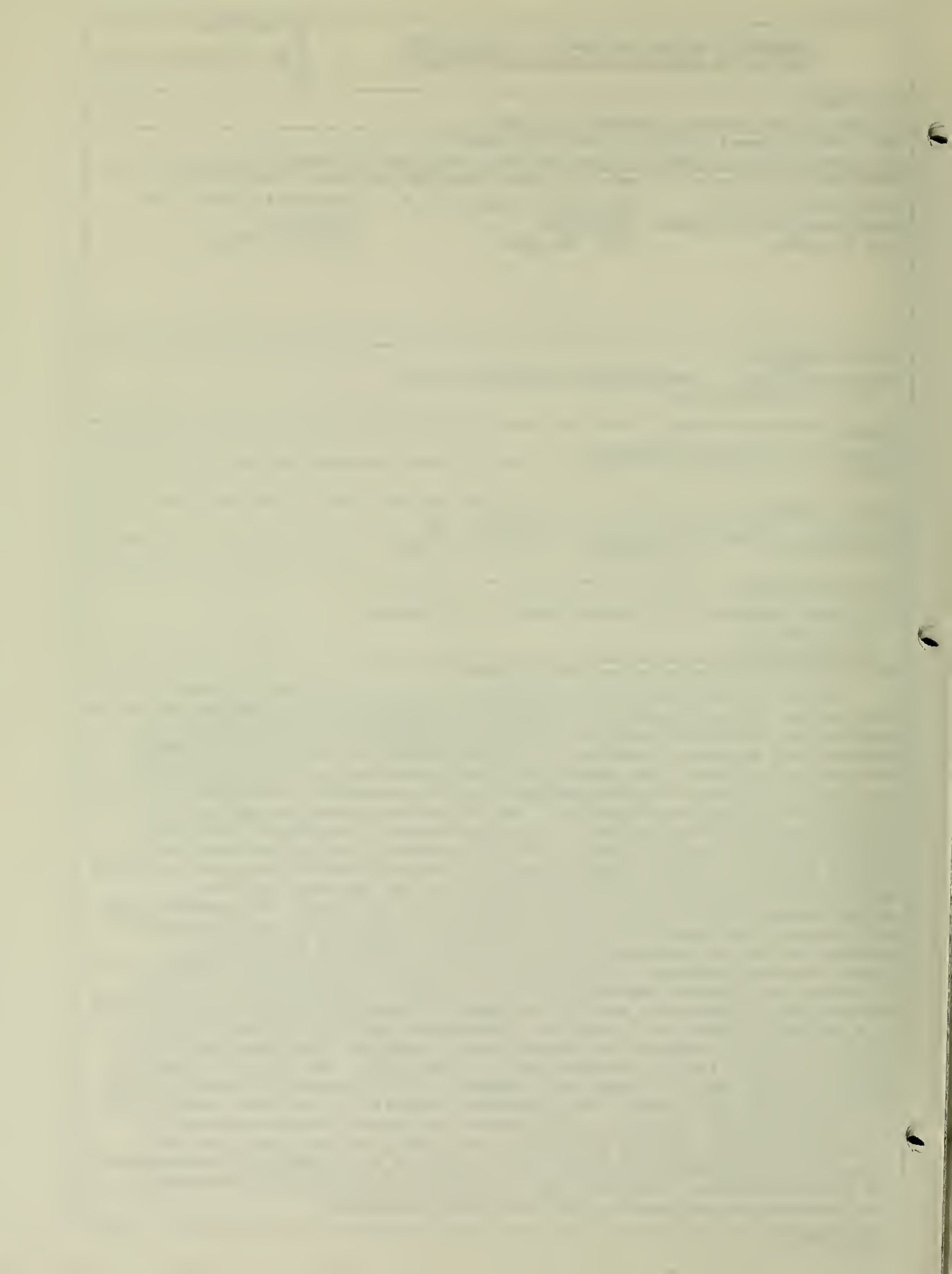
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Neutrophil mobilization to inflammatory sites is a multi-step process which involves the reversible adhesion to specific endothelial plasma membrane molecules, upregulation of selected neutrophil adhesion molecules during chemoattractant exposure, and migration (chemotaxis) through endothelial gaps and the basement membrane into tissues. The present study was undertaken to examine the changes that occur in adhesion molecule expression during neutrophil chemotaxis in vitro on a polycarbonate membrane substratum. The polycarbonate membrane system was selected for this study because: 1) unlike endothelial monolayers which have dynamic changes in their adhesion molecule expression, the polycarbonate surface remains relatively constant during neutrophil adherence and activation, and 2) two populations of neutrophils are easily identified and separated with the polycarbonate membrane system; one population does not migrate to chemoattractant and remains on the upper surface of the membrane, whereas the other population migrates through the membrane pores to the lower surface of the membrane (chemotactically responsive population). Neutrophils, incubated in suspension with or without the N-formyl peptide (FMLP) for 35 minutes, were compared with the chemotactically responsive and the nonresponsive subpopulations which were exposed to a gradient of FMLP for 35 minutes. Neutrophils were stained with a panel of adhesion molecule monoclonal antibodies which recognize: the leukocyte-cell adhesion molecule family (Leu-CAM), CD11a, CD11b, CD11c, and CD18; the platelet/endothelial cell adhesion molecule-1 (PECAM-1), CD31; leukosialin, CD43; and, the homing receptor Pgp-1, CD44. Flow cytometric analysis of the panel revealed that the chemotactically responsive subpopulation consistently down-regulated the expression of these adhesion molecules. CD44, CD43, and CD11a were the most dramatically down-regulated; CD11c, CD31, and CD11b were moderately down-regulated; and CD18 was slightly down-regulated. In contrast, only CD43 was consistently down-regulated on nonadherent, FMLP-stimulated neutrophils.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01034-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Generation and Characterization of CD45 Negative Macrophage Mutants

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

Liana Harvath, Ph.D.
Supervisory Microbiologist
LCH, DH, CBER

COOPERATING UNITS (if any)

Robert R. Aksamit, Lab of General and Comparative Biochemistry, NIMH
NIH, Bethesda, MD

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

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

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

The CD45 antigen family is a group of high molecular weight glycoproteins that are expressed on the plasma membranes of all leukocytes. CD45 has protein tyrosine phosphatase activity and appears to regulate signal transduction and lymphocyte activation by specific association with receptor molecules on T and B lymphocytes. We have previously shown that epitopes of CD45 are involved in neutrophil chemotaxis to the chemoattractants, leukotriene B4 and recombinant human C5a (rHuC5a). The purpose of this project is to generate and characterize stable CD45 negative myeloid cell clones and determine the role of CD45 in phagocyte function. The Abelson-murine leukemia virus transformed macrophage cell line, RAW 264, was selected for this project because RAW 264 macrophages exhibit dose-dependent chemotaxis to rHuC5a. RAW 264 cells were mutagenized with the alkylating agent, ethyl methanesulfonate, for 24 hours in tissue culture flasks. Mutagenized cells were scraped off the flask, replated into several flasks, and allowed to grow in Minimal Essential Medium containint 10% fetal calf serum. Cells were harvested from the flasks when they reached confluent growth and were treated with a biotinylated-monoclonal antibody (mAb) to murine CD45. Streptavidin-conjugated magnetic beads were incubated with the CD45 mAb-treated cells and the mixture was run over a magnetized column to enrich for the CD45 negative cells. After multiple rounds of CD45 negative selection and expansion of the enriched population in culture, 75 CD45 negative clones were identified by flow cytometric analysis. Twenty clones were screened for chemotactic responsiveness to rHuC5a and >90% of the clones had defective chemotactic responses. Current studies are in progress to transfect the CD45 negative mutants with CD45 cDNA and determine whether normal chemotactic function returns upon expression of CD45. These results indicate that CD45 expression on macrophage plasma membranes is important for normal chemotactic responsiveness to rHuC5a. The CD45 negative RAW 264 mutant clones provide a unique cellular system to probe the functional activity of CD45 in myeloid cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01006-08-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Flow Cytometry Facility

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

Douglas Terle, M.S.
Biologist
DH, CBER

Nicole Brownson, B.A.
Biologist
DH, CBER

Liana Harvath, Ph.D.
Research Microbiologist
DH, CBER

COOPERATING UNITS (if any)

Katie Pastakia, Ph.D.
Research Biologist
DH, CBER

Jeannette Ridge, Ph.D.
Research Biologist
DPQC,NLRC,CBER

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Laboratory of Cellular Hematology

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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The flow cytometry facility established seven years ago with the LCH has continued to provide service for a variety of clinical and basic research collaborative studies. Examples of representative projects conducted this year are outlined below.

1. Penicillin effects on agonist induced expression of platelet surface membrane glycoproteins. A collaborative study with Katie Pastakia and Kristina Prodouz evaluated expression of platelet glycoproteins when platelets were exposed to Penicillin G prior to thrombin activation. Fluorescein labelled antibodies to the glycoproteins were used to measure the effects of Penicillin G exposure. Results are in press (New York Academy of Science, Clinical Flow Cytometry, 1992).

2. Effects of nerve growth factor (NGF) and gamma-interferon (gamma-IFN) on subpopulations of the neuroblastoma cell line, SH-SY5Y. A collaborative study with J. Ridge and I. Levenbook examined the effects of NGF and Gamma-IFN, individuallly and in combination, on the growth, differentiation, and expression of specific antigens. Changes in antigen density and cell size during the course of NGF and gamma-IFN exposure were quantified with flow cytometry. Results were presented at 1992 World Congress on Cell and Tissue Culture, Washington, DC.

3. Characterization of adhesion molecule expression during chemotaxis. Quantitative changes in human neutrophil adhesion molecules have been evaluated with flow cytometry. The purified human neutrophils were separated into chemotactically responsive and nonresponsive subpopulations and the densities of a panel of antibodies to beta 2 integrins, platelet/ endothelial cell adhesion molecule-1, homing receptors and leukosialin. Results of the study were presented at the 1992 Armed Forces Institute of Pathology Qauntitative Histopathology Conference.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01007-04-LCH

PERIOD COVERED

October 1,1991 through September 30, 1992

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

Laser Scanning Confocal Microscopy Facility

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

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Supervisory Microbiologist
LCH, DH, CBER

COOPERATING UNITS (if any)

Aydin Tozeren, Ph.D. Dept. of Mechanical Engineering
The Catholic University of America, Washington, D.C.

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Laboratory of Cellular Hematology

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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.)

During the last four years, a laser scanning confocal microscopy facility has been established in the Laboratory of Cellular Hematology. Confocal microscopy is a state-of-the-art technology that enhances the contrast and resolution of microscopic images by rejecting out-of-focus information. Thin optical sections of a specimen are obtained, each image is stored on a computer, and the image series is reconstructed as a stereo or 3-dimensional image. The facility is currently used for the following projects:

1. The role of neutrophil cytoskeleton and integrin molecules on adherence strength. Adhesion strength of neutrophils to glass is quantified in a parallel plate flow chamber with a tapered gap width in which a constant shear stress gradient on the strength is evaluated in the presence and absence of: a) antibodies to integrin molecules, and b) cytochalasin D, an agent which inhibits elongation of the cytoskeletal protein, F-actin. F-actin is specifically stained with fluorescent phalloidin and the adherent cell F-actin network is imaged with the confocal microscope. Precise measurements of cell thickness and F-actin distribution are made under conditions of a constant shear stress gradient.

2. Myeloid membrane antigen location during leukocyte migration. Fluorescent monoclonal antibodies to neutrophil plasma membrane adhesion molecules are used to detect the location of the molecules on neutrophils that have migrated in vitro through polycarbonate membranes to chemoattractants. Results of this study were presented at the 1992 Armed Forces Institute of Pathology Quantitative Histopathology Conference.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-01024-02-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

INVESTIGATION OF THE PLATELET RESPONSE TO HYPOTONIC STRESS

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

Joseph C. Fratantoni, M.D.
Chief, Laboratory of Cellular Hematology
DH, CBER, FDA

Betty Poindexter, Biologist

COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

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

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

When platelets are suspended in hypotonic media, they swell, then slowly recover their original volume. This phenomenon has been utilized in an assay system in which platelets in plasma are diluted with water and the time course of the cell size changes followed by measurement of optical density. Recovery from this "hypotonic shock" has been found to correlate well with other viability measurements of platelets stored for transfusion. Yet, the cellular basis for this correlation, and for the recovery phenomenon itself, are not at all understood.

The usual method for performance of the assay involves diluting platelets in plasma in a cuvette and observing the optical density change with time. This is slow, cumbersome and severely limits attempts to correlate the volume changes with other cellular events. Further, Methods for calculating endpoints and expressing data are not at all standardized. We have adapted microtiter plate technology to the performance of hypotonic shock assays. This permits performance of 96 assays simultaneously and is a major advance from a technical point of view. We have investigated various approaches to calculation and display of data and have chosen to use a change of OD at 2 minutes as the principal variable. This permits statistical validation of variance and is a practical approach. We have begun to examine cytoskeletal protein changes at intervals after hyptonic challenge and to correlate these with cellular swelling, as determined by optical density. We will set up other methods for measurement of cell volume in order to verify the optical density readings.

Since the recovery response to hypotonic shock decreases with time in storage, we will also attempt to correlate the reaction with other events thought to be related to the storage lesion. One possibility is activation of platelet signal transduction mechanisms, especially those associated with transmembrane fluxes of calcium.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01016-03-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

MODIFIED HEMOGLOBINS AS A SOURCE OF ACTIVATED OXYGEN SPECIES

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

Abdu I. Alayash. Ph.D.
Sr. Staff Fellow

Beth A. Brockner Ryan
Biologist

Joseph C. Fratantoni, M.D.
Chief, Laboratory of Cellular Hematology

COOPERATING UNITS (if any)

Enrico Bucci, M.D., Univ. of Maryland, Baltimore, MD

Joseph Bonaventura, Ph.D. and Celia Bonaventura, Ph.D.,

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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.)

Hemoglobin-based oxygen carriers (HBOCs) are candidates for use as a blood substitute and resuscitation fluid. We have established that chemical modification used to generate hemoglobins cross-linked at either the or ß chains alter their ability to generate or interact with oxygen free radicals. Exposure of cross-linked hemoglobins to superoxide (O_2^-) generated by the xanthine/xanthine oxidase system causes generation of hydroxyl radicals (.OH) and/or other radicals with similar reactivity. Our results indicate that HBOCs exhibit a significant difference from each other and from unmodified hemoglobins in reactions involving the production of these oxygen free radicals. The relative ability of the ferric derivatives of HBOCs to participate in free radical reactions was monitored by nonenzymatic NADPH oxidation and aniline hydroxylation assays (reactions mediated by reactive oxygen species). Cross-linked hemoglobins again exhibited significant differences in their reactivity with oxygen species such as peroxides and superoxides. In more recent experiments, hydrogen peroxide was produced in a continuous flux, in order to mimic the cellular conditions, by the glucose/glucose oxidase system. Relative to unmodified HbA_0,we found that some cross-linked hemoglobins are more susceptible to oxidative modification and the formation of a highly toxic ferryl species. This form of oxidative modification may emerge as a physiologically important event which may lead to a significant contribution to reperfusion injury.

A number of in vitro and in vivo studies are planned in order to continue to examine the hemoglobin-mediated radical generation and to determine how these radicals may aggravate "reperfusion injury" in an ischemic animal model. Results of some of these studies were presented at the IV International Symposium on Blood Substitutes, 1991 in Canada. A manuscript describing some of the work has been published in Arch. Biochem. Biophys. Another manuscripts has been submitted for publication in Biochem. Biophys. Res. Comm.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH-01035-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

THE FORMATION OF ALTERED HEME PRODUCTS BY HUMAN HEMOGLOBINS

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

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Sr. Staff Fellow
DH, CBER

Beth Brockner Ryan
Biologist

COOPERATING UNITS (if any)

Yoichi Osawa, Ph.D., LCP, NHLBI, NIH
John Darbyshire, Ph.D., LCP, NHLBI, NIH

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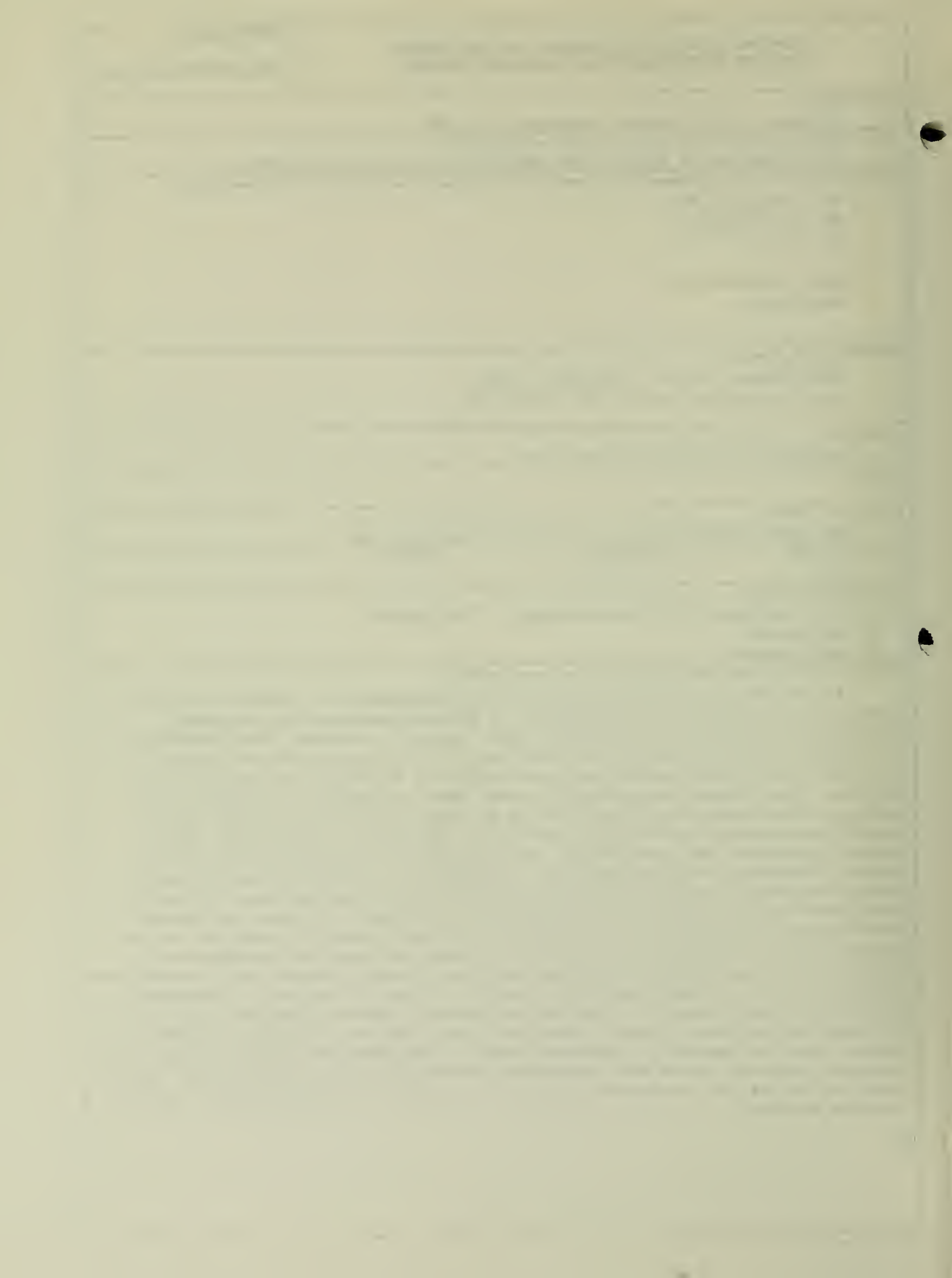
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

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

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

It is generally thought that oxidative modification by hydrogen peroxide or lipid peroxide lead to the inactivation of various hemoproteins, including myoglobin, hemoglobin and cytochrome P-450. Recently, however, work reported by Dr. Y Osawa at the NIH showed that addition of 1-2.5 equivalents of hydrogen peroxide to myoglobin resulted in cross-linking of an intact heme moiety to the protein. This altered hemoprotein has been shown to exhibit an oxidase-like activity. The alteration of myoglobin to an enzyme that can form toxic oxygen metabolites may have pathological importance, especially in myocardial injury caused by ischemia and reperfusion. This prompted us to ask the question of whether chemically modified hemoglobins developed as blood substitutes undergo similar structural and/or enzymatic modifications which may explain, at least in part, some of the toxicities associated with the infusion of hemoglobin-based products such as vasospasm and reoxygenation injury. Results transpired so far from our recent collaborative work with Dr. Osawa indicate that treatment of hemoglobin with low levels of hydrogen peroxide produced soluble protein-bound heme products that are chromatographically similar to that of myoglobin. Moreover, hemoglobins cross-linked at alpha subunits showed a typical propensity to oxidative modification than other forms of hemoglobin modifications. The heme derived adducts from the reaction of hydrogen peroxide with hemoglobin showed little or no enzymatic activity in the NADPH-diaphorase methemoglobin reductase system. Work is under way to test for enzymatic activity in a variety of physiologically relevant reducing systems.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01028-02-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

INTERACTIONS OF MODIFIED HEMOGLOBINS WITH IRON CHELATORS

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

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0.3	0.3	

CHECK APPROPRIATE BOX(ES)

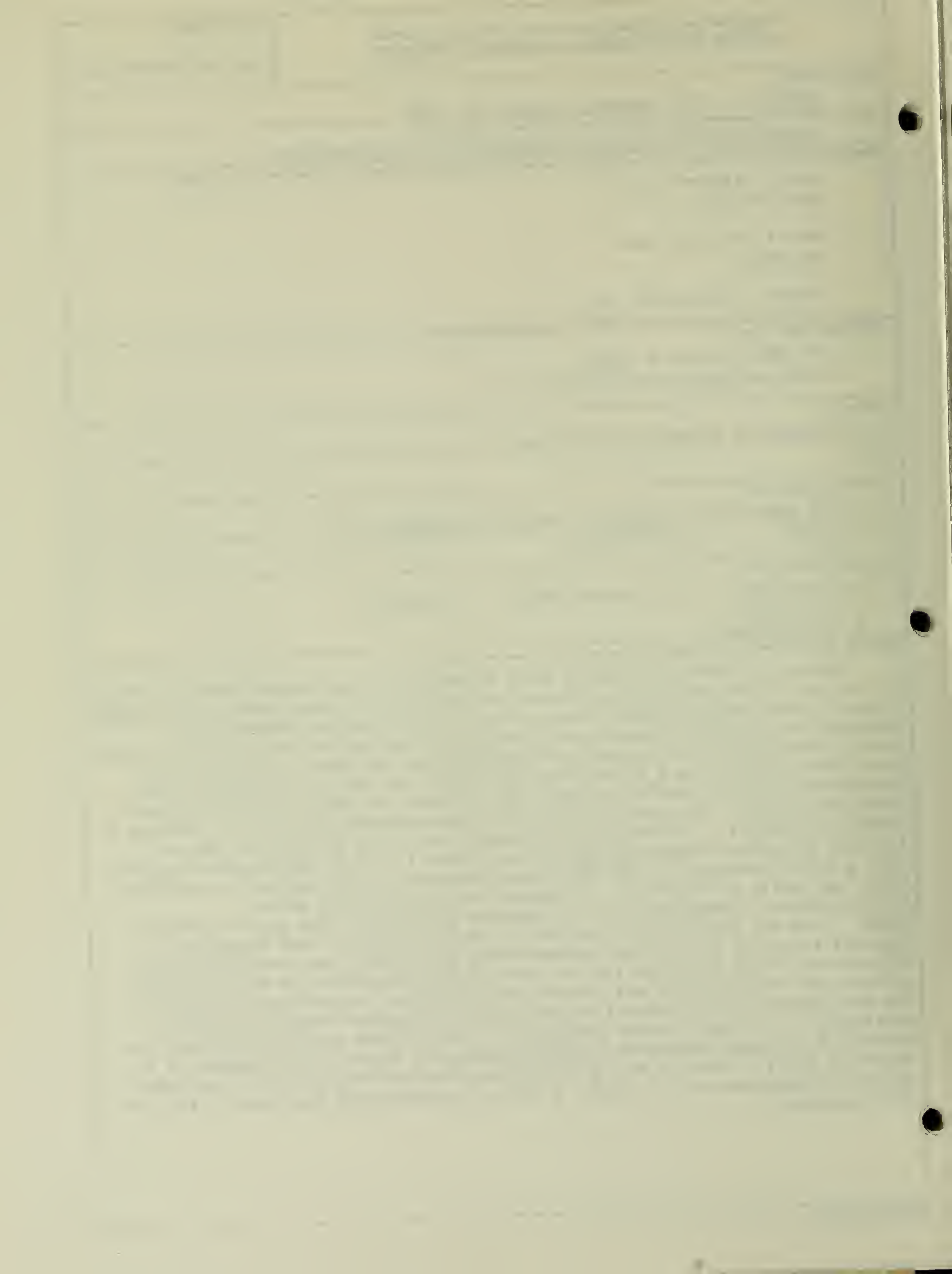
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Among the current strategies aimed at decreasing the oxidant effect of HBOCs is the use of iron chelators to prevent the potential molecular and cellular damage caused by iron induced oxygen free radicals. We studied the effects of interactions of desferrioxamine and a newly developed pyrone, ethyl maltol, on the rate of autoxidation and hydrogen peroxide mediated oxidation of cross-linked hemoglobins. There was a slight decrease in both the rate of autoxidation and hemichrome formation when ethyl maltol was included in the solutions containing these hemoglobins. Conversely, inclusion of desferrioxamine led to an increase in oxidation products. In the reaction mixture and in the presence of excess hydrogen peroxide, desferrioxamine led to a 20-30 % rise in methemoglobin coupled with a slight reduction in the hemichrome formation. Ethyl maltol, on the other hand, has little or no effect. It appears that unlike ethyl maltol, desferrioxamine, independent of its chelating properties, acts as an oxidizing agent. This may relate to its ability to interact directly with oxygen free radicals resulting in increased methemoglobin formation. The observed relative differences in the susceptibility of cross-linked hemoglobins to the prooxidant activity of desferroxamine may reflect the differences in the stereochemistry of the heme pocket of the proteins brought about by different forms of chemical modification. Further studies are planned to verify this and to investigate the underlying molecular mechanism of desferrioxamine's action. Also planned is a follow up study in which a more powerful and effective family of iron chelating pyridinone compounds will be used. A manuscript describing this work in full is being prepared.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01036-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

NITRIC OXIDE BINDING TO CROSS-LINKED HUMAN FERRIHEMOGLOBINS

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

Abdu I. Alayash. Ph.D.
Sr. Staff Fellow

Joseph C. Fratantoni, M.D.
Chief, Laboratory of Cellular Hematology
DH, CBER, FDA

COOPERATING UNITS (if any)

Robert E. Cashon Ph.D., Joseph Bonaventura, Ph.D.
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0.4	0.4	

CHECK APPROPRIATE BOX(ES)

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

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

Intravascular administration of stroma-free hemoglobin appears to be strongly correlated with a rise in pulmonary and systemic blood pressure. This hypertensive response has been attributed to interactions between hemoglobin and the endothelium-derived relaxing factor (EDRF), which is now believed to be nitric oxide (NO). We have explored the interactions between NO and oxidized human hemoglobin, comparing the behavior of unmodified HbA_0 with that of two chemically cross-linked hemoglobins. The latter are promising blood substitute candidates due to their lower oxygen affinity and greater stability of tetrameric structures. The modified forms examined were HbA-DBBF, cross-linked between the alpha chains with bis (3,5-dibromosalicyl) fumarate, and HbA-FMDA, modified between the beta chains with fumaryl mono-dibromoaspirin. Biphasic kinetics of NO binding to the ferric hemes were observed, attributable to differing reactivities of alpha and beta chains. The rates of the two phases were altered in the modified hemoglobins, indicating that interdimeric cross-linking changes both alpha and beta chain heme pockets. In a much slower subsequent process, the ferric hemes of (Fe^{III}-NO) complex became reduced, as shown by appearance of the (Fe^{II}-NO) spectrum. The reduction occurred at differing rates for the hemoglobins studied, with the fastest time courses shown by the modified forms. If the vasoconstrictive effects of extracellular hemoglobin involve cycling between oxidized and reduced forms in response to exposure to NO, the differences in rate constants observed for the modified hemoglobins may prove to be of physiological significance.

Results are compiled in a manuscript submitted for publication

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01026-02-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

TEMPERATURE AFFECTS O_2-CARRYING OF CROSS-LINKED HEMOGLOBINS

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

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Beth A. Brockner Ryan
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Joseph C. Fratantoni, M.D.
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0.3	0.3	

CHECK APPROPRIATE BOX(ES)

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

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

In view of the potential application of HBOCs in perfusion of organs for cardioplegia during major surgery, or for preservation of donated organs at low temperatures before transplant, we examined temperature dependence profiles of oxygen equilibrium curves (OECs) at 15-37 °C for a number of human and bovine cross-linked hemoglobins. OECs for these hemoglobins were carried out on the Hemox-Analyzer fitted with a thermostated cell. OECs for modified hemoglobins at 37 °C were right shifted as compared to unmodified hemoglobin. Lowering the temperature however, resulted in increased affinity towards oxygen. It shifted OECs to the left. However, bovine hemoglobin cross-linked at the ß-ß subunits exhibited significantly different and more favorable oxygenation at very low temperature. Such is the case with a newly developed modified human hemoglobin cross-linked with a pyridoxyl tetraphosphate derivative. This led us to believe that not only the site of modification, but also the nature of the chemical modification plays a crucial role in determining the degree of conformational constrains placed upon the hemoglobin when loading and unloading oxygen. Kinetic characterization of ligand binding to these hemoglobins is now underway using fast reaction techniques in an attempt to relate both equilibrium and kinetic manifestations of hemoglobin function under hypothermic conditions. Part of this work has been published in Biomat. Art. Cell Immob Biotech (1992). A short communication describing this work fully has been prepared for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01012-03-LCH

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

THE EFFECT OF PENICILLIN G ON PLATELET MEMBRANE GLYCOPROTEINS

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

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Staff Fellow

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Chief, Laboratory of Cellular Hematology

Kristina N.Prodouz, Ph.D
Research Chemist

Douglas Terle, M.S.

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0.5	0.5	

CHECK APPROPRIATE BOX(ES)

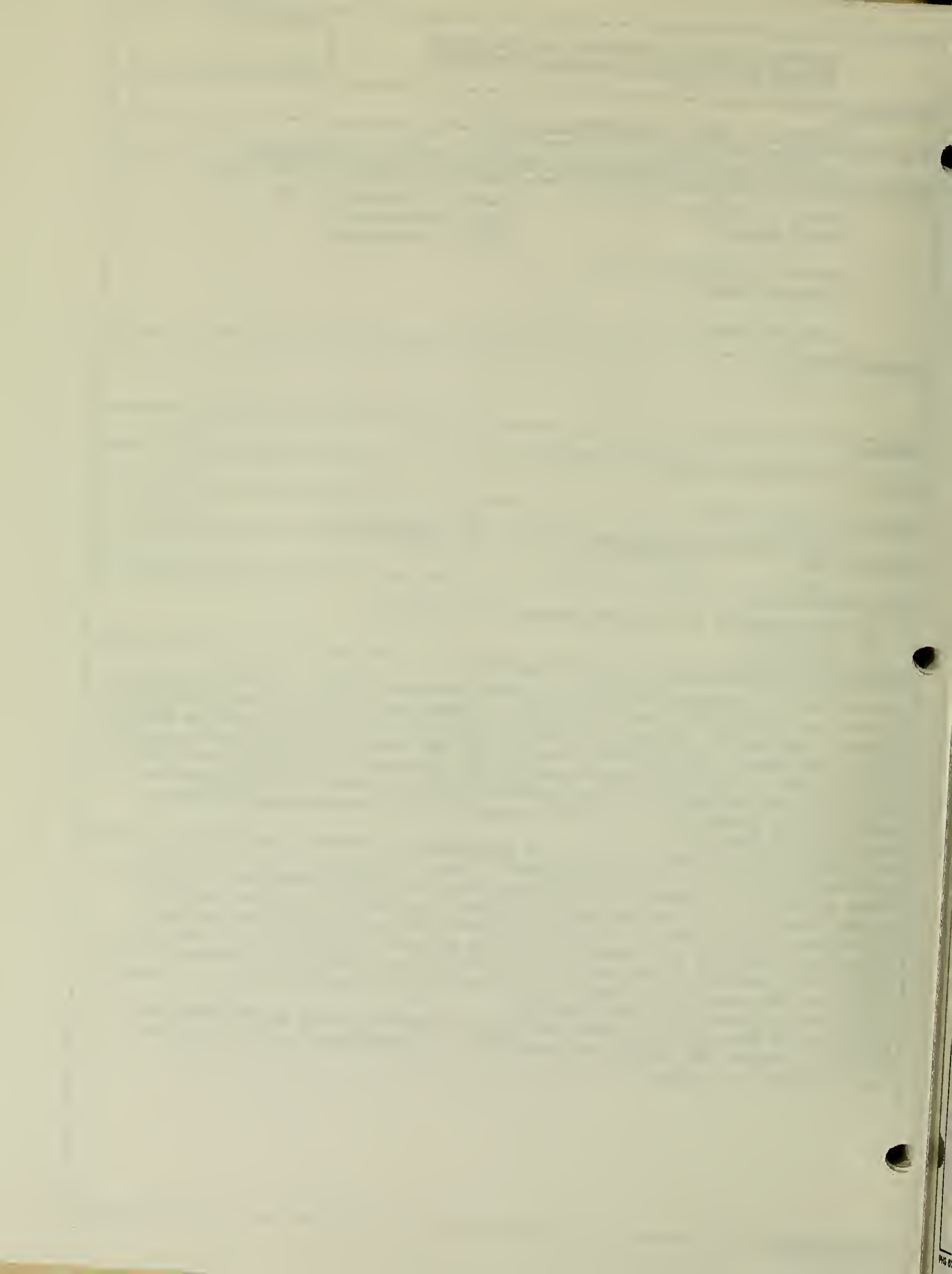
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Penicillin G, a β-Lactam antibiotic, induces abnormalities in platelet function, in part, by inhibiting platelet membrane receptors and, also, by inhibiting post-receptor biochemical events. The platelet surface is the primary site of exposure to the antibiotic. We therefore investigated the effect of penicillin on platelet function and on the surface expression of the membrane glycoproteins GPIb, GPIb-IX, GPIIb-IIIa and P-selectin by flow cytometry and monoclonal antibodies directed against these proteins. In initial studies, platelet concentrates exposed to penicillin (2-20mM) for
48 h showed irreversible inhibition of aggregation by thrombin ($\leq$0.4U/ml) in washed platelets after removal of the antibiotic. Brief exposure (15 min) to similar doses of penicillin also inhibited aggregation but it was reversible upon removal of the drug. Flow cytometric analysis using fluorescently-labelled monoclonal antibodies revealed that brief (15min) exposure of washed platelets to 10mM penicillin inhibited the thrombin-modulated decrease in expression of GPIb and GPIb-IX and increase in expression of GPIIb-IIIa and P-selectin. Penicillin also inhibited the regulation of GPIb-IX and GPIIb-IIIa surface expression in platelets in plasma activated by thrombin and ADP.
A portion of this study has been published (Sloand EM, Klein HG, Pastakia KB, Pierce P, Prodouz KN. Blood 1992:2022-2027). A second manuscript, submitted to J Lab Clin Med, is in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01010-06-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

INACTIVATION OF VIRUSES IN BLOOD PRODUCTS BY INHIBITION OF VIRAL NUCLEIC ACID

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

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0.8	0.8	

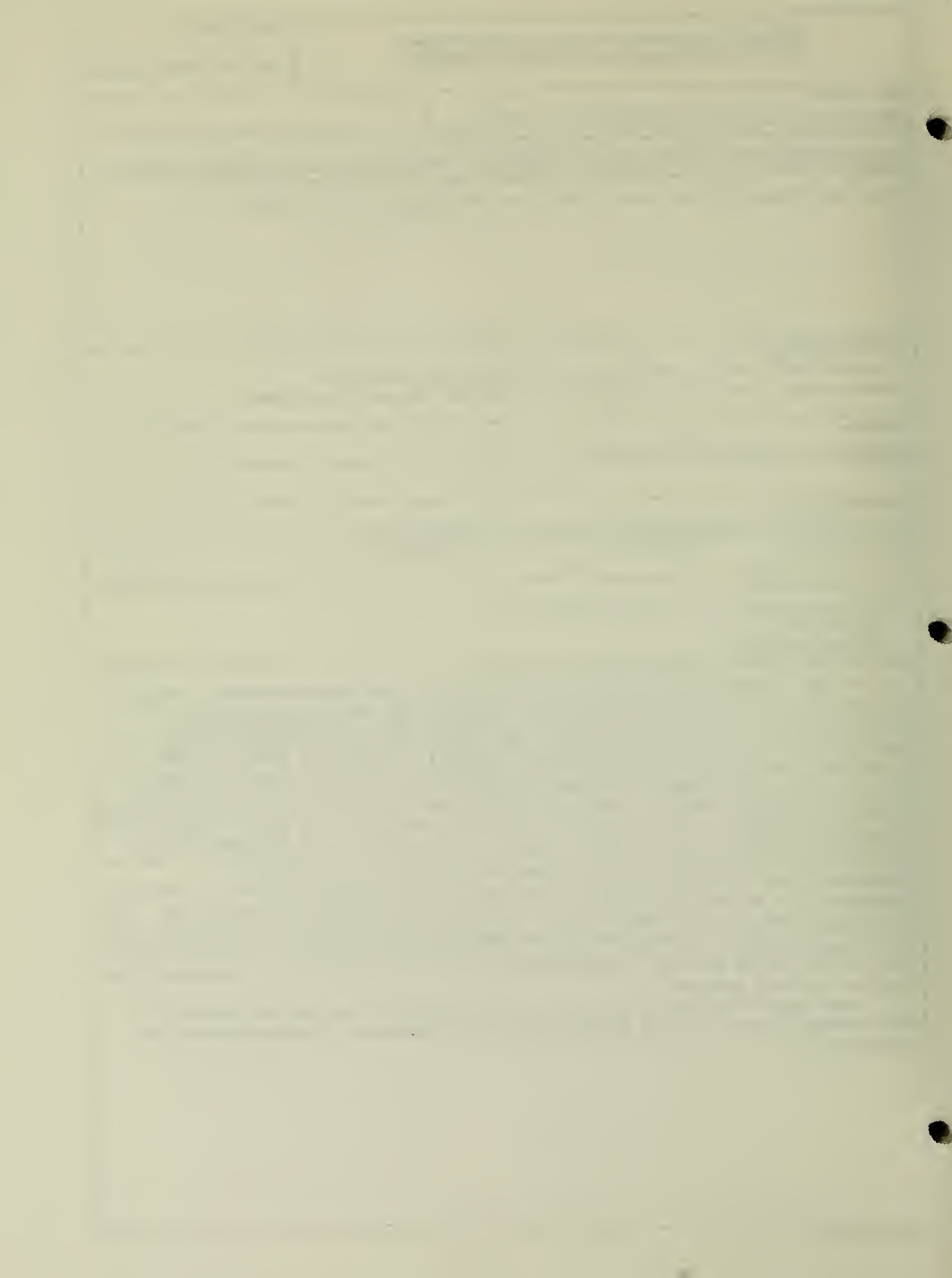
CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues X
  ☐ (a1) Minors
  ☐ (a2) Interviews

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

We have investigated the antiviral activity of the photoactivatable compound gilvocarcin (GV), an antitumor agent which is closely related to psoralen. GV binds to DNA in the presence of near UV radiation (UVA, 320-400nm) through cycloaddition, blocks DNA replication, and causes DNA strand breaks. GV has been shown to be toxic to bacteria and mammalian cells at picomolar levels in the presence of UVA. GV was considered to be an ideal treatment of the inactivation of viruses which contaminate transfusable blood products, since viral nucleic acid would be targeted, thereby eliminating most damage to platelets and red blood cells which are anucleate. We evaluated the effectiveness of GV and UVA for inactivation of several viruses, including the bacterial viruses ΦX174, T7, PRD1 and Φ6, and herpes simplex virus, type 1 (HSV). Some inactivation of the bacterial viruses was observed with UVA radiation alone (exposures ≤ 26 kJ/m^2). Additional, photosensitized inactivation was observed only with Φ6 and T7 at 2uM GV. HSV was photosensitive at concentrations of GV three orders of magnitude lower (1nM). The survival curves for all three viruses displayed multicomponent kinetics, indicating that 80-90% of these viruses constituted sensitive populations). At present there is no explanation for the wide range of sensitivities. The lack of inactivation of two viruses, ΦX174 and PRD1, suggests that GV is not appropriate to photoinactivate viruses in blood products.

These data were presented at the annual meeting of the American Society for Photobiology, June, 1991. A manuscript will be submitted to Photochemistry and Photobiology.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01009-04-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

EFFECT OF THE PHOTOSENSITIZER MEROCYANINE 540 ON PLATELET MEMBRANES

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

Kristina Prodouz, Ph.D., Research Chemist, LCH, DH, CBER, FDA
Joseph C. Fratantoni, M.D., Chief, Laboratory of Cellular Hematology
Elizabeth Keville, Chemist, LCH

COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER.
0.7	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 lipophilic photosensitizer, merocyanine 540 (MC) has been proposed for use as an agent to reduce the transmission of virus by cellular blood components. In a previous study, we demonstrated the antiviral activity of MC in the presence of visible light (450-600nm). The inactivation of lipid-enveloped viruses by dye and light is most likely mediated by reactive singlet oxygen. Platelets are highly susceptible to photodamage by MC and demonstrate marked morphological alterations, a disruption of the normal response to agonists, and a spontaneous release of granule contents.

Significant photodamage to the platelet membrane upon treatment with dye and light has been demonstrated in studies of MC binding to membranes in the absence of presence of exogenous albumin, by measurement of arachidonic acid release from membranes, and by SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analyses of alterations of the migration of membrane proteins following treatment. In addition, MC ± light caused the generation of microvesicles (MV) from membranes, indicating activation of platelets. Patterns of MV protein composition were analyzed by SDS-PAGE and were found to be significantly different in MV generated with MC + light vs. minus light. Individual proteins incorporated into MV were identified by immunoblotting using antibodies to major platelet glycoproteins, cytoskeletal proteins and granule proteins. In addition, MC ± light caused the formation of a high molecular weight membrane protein complex which has been analyzed by size exclusion chromatography and 2-dimensional isoelectric focusing and SDS-PAGE.

Results of this study were presented at the annual meeting of the American Society for Photobiology in June, 1991 and were published in Blood Cells, in December, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01037-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

INDUCTION OF CALPAIN ACTIVITY DURING STORAGE OF PLATELET CONCENTRATES

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

Kristina Prodouz, Ph.D., Research Chemist, LCH, DH, CBER, FDA
Elizabeth Keville, Chemist, LCH, DH, CBER, FDA

COOPERATING UNITS (if any)

Edward Snyder, M.D., Yale-New Haven Hospital, New Haven, CT

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

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

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

During blood bank storage of platelet concentrates (PC), platelet actin is hydrolyzed into two fragments (SP-1, 29kd and SP-2, 27kD). To determine if calpain-induced proteolysis was responsible for degradation of actin, we analyzed cytoskeletal proteolysis in intact platelets obtained from units of PC stored under blood bank conditions for up to 10 days. PC were incubated with 0.9% NaCl (control); calcium ionophore A23187 (calpain agonist); and leupeptin and E64d (calpain inhibitors). SDS-PAGE and immunoblotting was performed with probes for actin binding protein (ABP), talin, vinculin and glycoprotein IIb. Results showed that during blood bank preparation and storage, actin, ABP, talin and vinculin were degraded with concomitant generation of specific fragments over time. In contrast, glycoprotein IIb levels were unchanges during the storage period. Degradation of cytoskeletal proteins was enhanced by exposure of PC to A23187, and inhibited by incubation of PC with E64d. These results imply that calpain is involved in this degradative process and that cytoskeletal proteolysis may play a role in the development of the platelet storage lesion.

Results of this study were presented at the annual meeting of the American Society for Hematology, December, 1991 and have been prepared for submission to BLOOD.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01038-01-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

PRESENCE OF CALPAIN-INDUCED ACTIN FRAGMENTS IN PLATELET MICROVESICLES

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

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Elizabeth Keville, Chemist, LCH, DH, CBER, FDA

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0.4	0.4	

CHECK APPROPRIATE BOX(ES)

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

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

We are evaluating whether the generation of microvesicles (MV) known to occur during storage of platelet concentreates involves calpain-induced proteolysis of cytoskeletal proteins, including actin. MV, which are shed when calpain is activated by physiological agonists, may be the result, in part, of proteolytic cleavage of actin-binding protein (ABP) by calpain at the actin-membrane interface. During blood bank storage of platelet concentrates (PC), platelet actin is hydrolyzed into two fragments (SP-1, 29kd and SP-2, 27kD) and this cleavage may also be due to calpain activation. We analyzed cytoskeletal proteolysis in MV obtained from units of PC stored under blood bank conditions for up to 10 days. PC were incubated with 0.9% NaCl (control); calcium ionophore A23187 (calpain agonist); and leupeptin and E64d (calpain inhibitors). MV were prepared from PC by high speed centrifugation. SDS-PAGE and immunoblotting was performed with probes for actin binding protein (ABP), talin, vinculin and glycoprotein IIb. In addition, the MV pellet was solubilized in urea for 2D-IEF/SDS-PAGE (2D-PAGE). Results showed that during blood bank preparation and storage, MV actin, ABP, talin and vinculin were degraded with concomitant generation of specific fragments over time. Glycoprotein IIb levels increased in MV during the storage period which indicated that MV generation increased with time. Degradation of MV actin was enhanced by exposure of PC to A23187, and inhibited by incubation of PC with E64d. These results imply that calpain activation occurs in PC during storage and that activation increases over time. Preliminary results suggest that shear stress may contribute to MV generation. Cytoskeleton disruption by cytochalasin D or vinblastine was not sufficient to cause MV release or calpain activation.

Initial results of this study were presented at the annual meeting of the American Society for Hematology, ~~December, 1991. A manuscript is in preparation.~~

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 01013-03-LCH

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

DEVELOPMENT OF NEW ASSAYS TO EVALUATE FUNCTION OF STORED PLATELETS

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

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Joseph Fratantoni, M.D., Chief, LCH, DH, CBER
Katie Pastakia, Ph.D., Staff Fellow, LCH, DH, CBER
Elizabeth Keville, Chemist, LCH, DH, CBER

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0.8	0.8	

CHECK APPROPRIATE BOX(ES)

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

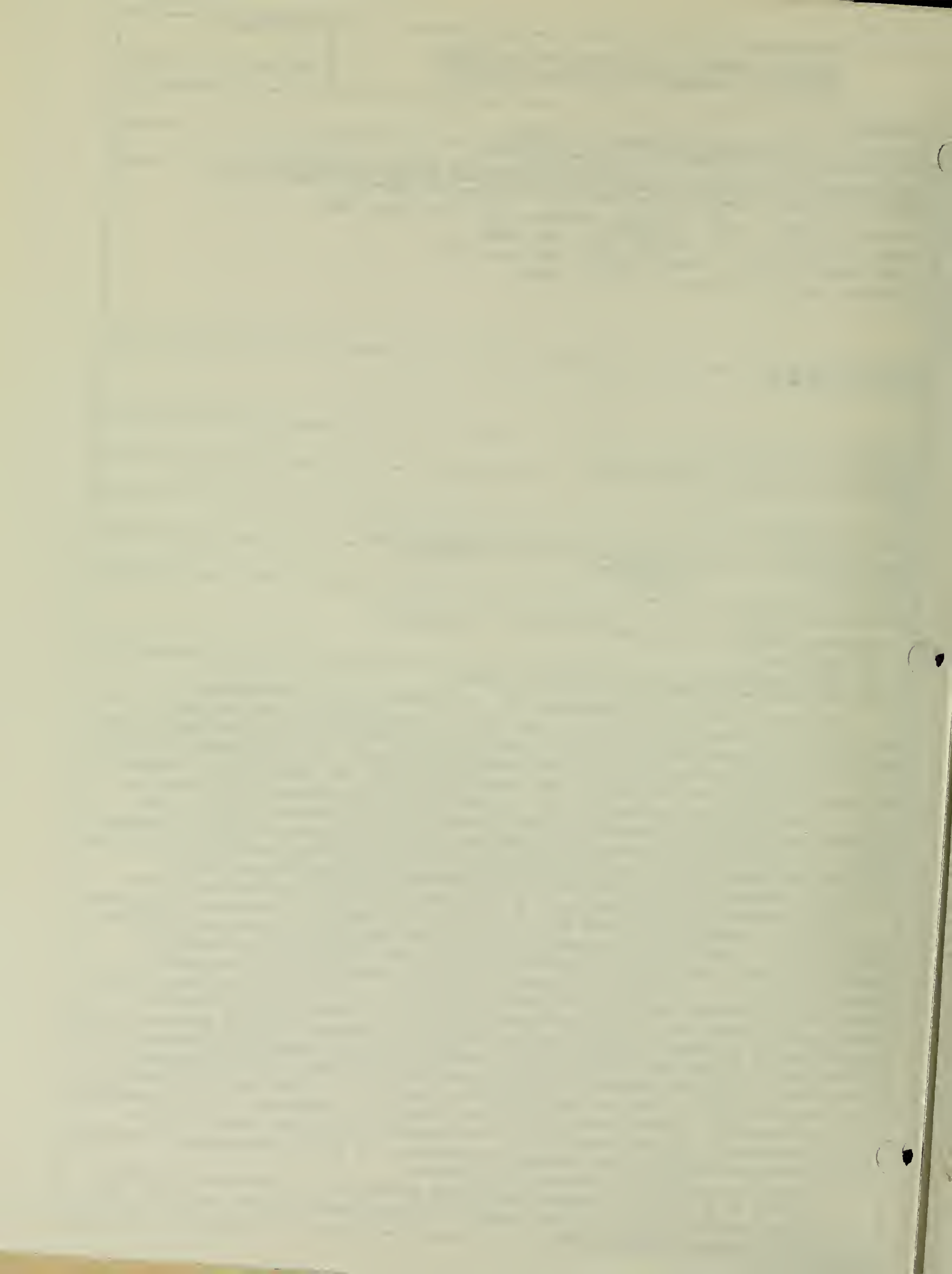
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The overall quality of platelets for transfusion is not considered satisfactory by most researchers in the field. The problem is due, in part, to the difficulty of collecting and storing these highly reactive cells, and also to the lack of adequate in vitro methods for assessing quality. We have sought to bring methods of modern cell biology to the investigation of the cellular and biochemical basis of the platelet storage lesion. We have available several very sensitive methods for identifying storage-induced changes in the platelet membrane, the membrane skeleton and cytoskeletal proteins. Characterization of protein patterns has been achieved by one- and two-dimensional SDS-PAGE and by immunoblotting using a variety of monoclonal antibodies to cytoskeletal and membrane proteins.

Flow cytometry has proven to be a very sensitive technique for monitoring activation of platelets and the mobilization of glycoprotein receptors in response to agonists. A method for analysis of activation-dependent changes in platelets sampled directly from platelet concentrates has been developed and includes formalin-fixation of platelets diluted in plasma. Washing steps have been eliminated from this procedure, allowing platelets to be analyzed directly without centriguation which may cause additional activation. Fluorescently-labeled antibodies to P-selectin (CD62) and p53 (CD63), a lysosomal granule protein, allow rapid, direct detection of activation. Regulation of glycoprotein expression under conditions of agonist-induced activation has been monitored with antibodies to GPIb, GPIb/IX complex, GPIIb, and GPIIb/IIIa complex. In addition, the amount of filamentous actin, another indicator of activation, is measured with the sensitive probe, rhodamine-labelled phalloidin. The platelet activation state will be correlated with morphological changes demonstrated by phase microscopy and with any alterations in aggregation response to agonists.

Results of a platelet storage study were presented at a symposium - "The Celular and Molecular Basis of the Platelet Storage Lesion", held in April, 1991 and were published in March, 1992.

PHS 6040 (Rev. 5/92)



ANNUAL REPORT

Hemostasis and Thrombosis Laboratory

October 1, 1991 through September 30,1992

The Hemostasis and Thrombosis Laboratory maintains active programs in research and regulatory review. There are currently three investigators pursuing basic research projects in different areas of hemostasis and thrombosis. These areas include factor VIII/von Willebrand factor, tissue factor pathway inhibitor, and regulation of urokinase expression by tumor cells. In addition, there are several ongoing projects on product characterization and standardization. Finally, the Laboratory devotes a considerable proportion of its resources to review of product and establishment applications/amendments, INDs, and other regulatory activities.

Research Activities

Dr. Lewis's research has focused on tissue factor pathway inhibitor (TFPI), which is a circulating plasma protein that binds to coagulation Factor Xa (F Xa) and inhibits F Xa activity. This bimolecular complex can then bind to the Factor VIIa-tissue factor complex (VIIa-TF) and, in so doing, inhibit the Factor X (F X) and Factor IX (F IX) -activating ability of VIIa-TF. Immunochemical studies have focused on the ability of both monoclonal antibodies and rabbit polyclonal antibodies to neutralize the various activities of the TFPI. Assays were developed to measure the TFPI inhibitory activity toward Xa and VIIA-TF and to measure TFPI antigen. Polyclonal antisera were used to investigate the contribution of TFPI in the prothrombin time clotting assay in normal, hemophilia A and hemophilia B plasmas. The TFPI assays were also applied for the identification of monoclonal antibodies specific for the particular functional epitopes.

Dr. Silverman, who has been with the Laboratory for about a year, is studying the regulation of plasminogen activator inhibitor - 1 (PAI-1) and of urokinase plasminogen activator (uPA) expression in three osteosarcoma cell lines. The three cell lines, which have different metastatic potential, have differing levels of expression of PAI-1 and uPA. Those with higher uPA mRNA expression also have higher metastatic potential. There also appear to be differences in regulation of PAI-1 in these cells. Early data suggest that the 5' flanking region of uPA contains a negative regulatory element active in the osteosarcoma cells with lower metastatic potential, but that the same region confers positive promoter activity in the cell lines with higher metastatic potential.

Dr. Fricke has focused his efforts on epitope mapping of human antibodies to factor VIII and von Willebrand factor. Thrombin digestion of factor VIII followed by immunoprecipitation with

antibodies occurring in two patients has demonstrated that one patient has an antibody that recognizes the light chain only, while the other patient has antibodies that recognized the light chain, and the A1 and A2 regions of the heavy chain. Digestion of factor VIII with trypsin has been been optimized, but binding of the antibodies to the digested factor VIII has not been sucessful. Preliminary experiments with trypsin digestion of von Willebrand factor have been done, and the conditions optimized. The immunoprecipitation experiments are being planned.

A variety of product characterization and standardization studies are in progress. The study of standards for coagulation factor IX was published in Thrombosis and Haemostasis in December. That study demonstrated that a factor IX complex type standard could be used to assign potency to Coagulation Factor IX products. A large, collaborative study designed to address the question of assigning potency to von Willebrand factor concentrates was completed. The results have been presented at the American Society of Hematology meeting and at the Scientific and Standardization Committee meeting of the International Society on Thrombosis and Haemostasis. The paper describing the study is under review. This study showed that there is considerable variability between labs and between assay types for vWF:Ag and ristocetin activity. However, it also showed that plasma could be used as a standard because only a few assays were not valid due to nonparallelism with the standard.

The Seroconversion Surveillance Project, a collaborative study involving the FDA, National Hemophilia Foundation, and the Centers for Disease Control, is in its last year. The Project, designed to monitor users of clotting factor concentrates for evidence of transmission of HIV, has investigated forty-four reported HIV seroconversions since 1987. Nine of these were accepted as meeting the CDC criteria for probable association of HIV seroconversion with virus-inactivated clotting factor concentrate. However, none were due to concentrates that are or were on the market at the time of seroconversion.

Regulatory Activities

The Laboratory has continued to struggle with a large regulatory load and chronic understaffing. The Laboratory received approximately 322 IND submissions for review, including 20 original submissions. There were 28 approvals of product and establishment applications and amendments, and 26 new product and establishment applications and amendments were received during the fiscal year. The current backlog of applications and amendments under review is 75. Personnel from the Laboratory conducted inspections at seventeen licensed facilities.

Publications

Silverman TA, Noguchi M, Safer B. Role of sequences within the first intron in the regulation of expression of eukaryotic

invitation factor 2α. J Biol Chem 267:9738-9742m, 1992.

Lamb MA, Fricke WA, Rastogi SC: Standardization of Factor IX: Standards for "Purified" Factor IX Concentrates. Thromb Haemost 66:548-551, 1991.

Fricke WA, Augustyniak L, Lawrence D, Brownstein A, Kramer A, Evatt B: Human immunodeficiency virus infection due to clotting factor concentrates: Results of the seroconversion surveillance project. Transfusion (in press).

Abstracts

Lamb M, Fricke W, Rastogi S: Potency of von Willebrand factor concentrates: An international collaborative study. Blood 78:264a, 1991.

Lewis RM, Schneider MJ, Fricke WA: Neutralization of tissue factor pathway inhibitor corrects the clotting time of hemophilic plasma in vitro. Blood 78:281a, 1991.

Fricke W, Koo E, Schneider M, Lewis R: Activation of factor VIII by factor Xa is blocked by tissue factor pathway inhibitor. Blood 78:1922a, 1991.

Fricke W, Lebel D, Koo E: Structure of factor VIII in factor VIII concentrates. Transfusion (accepted).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 BH 07002-02-LHT

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Standards for assay of von Willebrand factor: A collaborative study

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

William Fricke, MD
Mary Ann Lamb, PhD
Suresh Rastogi, PhD

COOPERATING UNITS (if any)
Division of Biostatistics and Epidemiology

LAB/BRANCH
Hemostasis and Thrombosis Laboratory

SECTION
Division of Hematology

INSTITUTE AND LOCATION
Center for Biologics Evaluation and Research

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.3	.3	0

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

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

A multilaboratory collaborative study was undertaken to assess the feasibility of using a plasma standard for assaying the von Willebrand factor content of von Willebrand factor concentrates and of factor VIII concentrates. Thirteen laboratories tested six concentrates for von Willebrand factor antigen, ristocetin cofactor activity, and multimer content using the World Health Organization plasma standard for factor VIII/von Willebrand factor, 87/718, as a standard. Only a few assays were invalid because of nonparallelism or nonlinearity. Significant interlaboratory and interassay differences were found for both von Willebrand factor antigen and ristocetin cofactor activity. There was generally good agreement between the laboratories with respect to multimer content in the preparations. With respect to assay validity, a plasma standard could be suitable for assaying concentrated preparations of von Willebrand factor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 07003-02-LHT

PERIOD COVERED

October 1, 1992 to September 30, 1992

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

Characterization of the factor VIII in factor VIII concentrates

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

William Fricke, MD
Don Lebel, BS
Eleanor Koo, MS

COOPERATING UNITS (if any)

none

LAB/BRANCH

Hemostasis and Thrombosis Laboratory

SECTION

Division of Hematology

INSTITUTE AND LOCATION

Center for Biologics Evaluation and Research

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.3	.1	.2

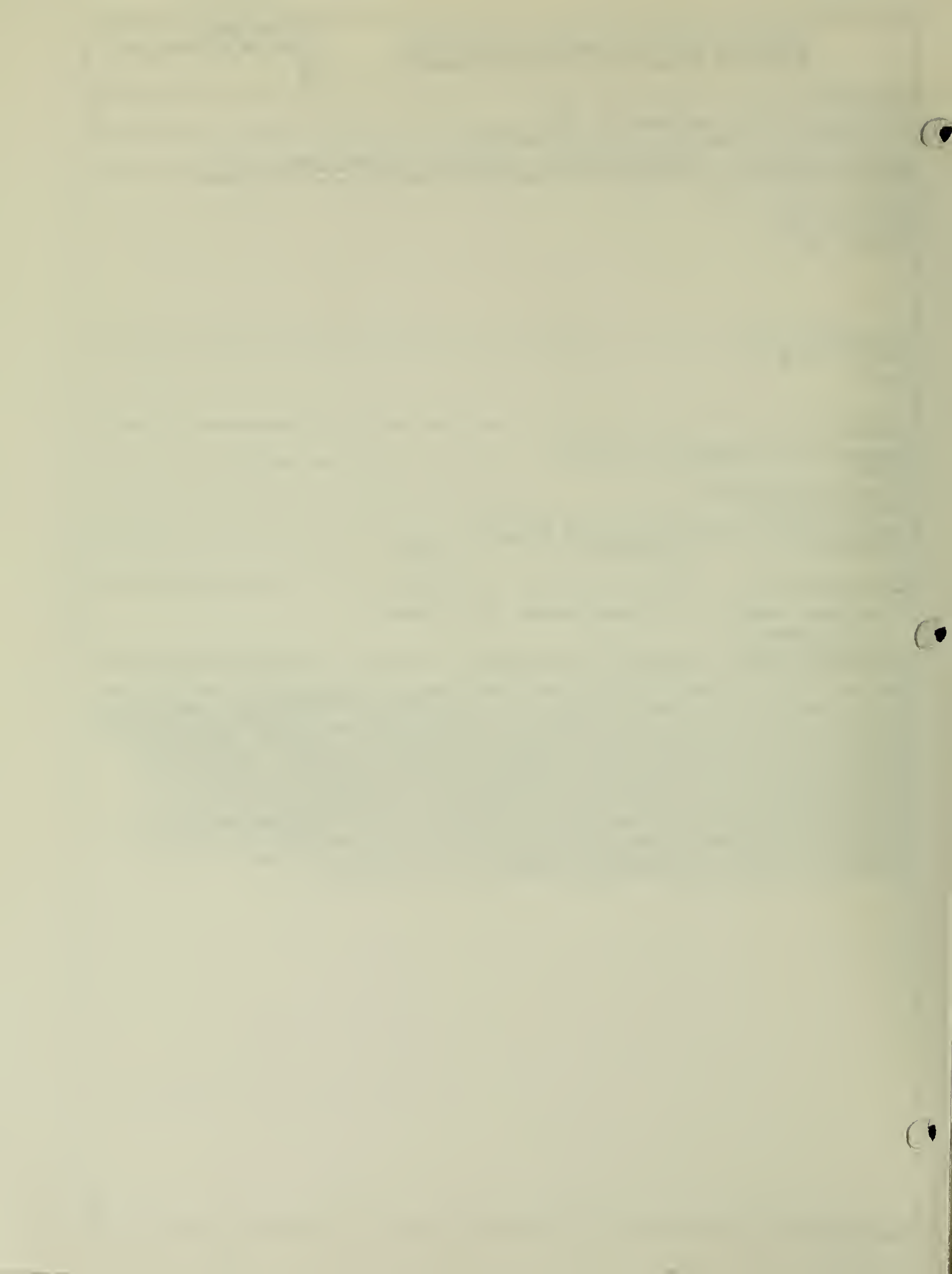
CHECK APPROPRIATE BOX(ES)

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

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

The factor VIII in factor VIII concentrates is being characterized by activity and antigen assays and by immunoblotting. The availability of factor VIII concentrates of increasing purity, including recombinant factor VIII, has raised concern about potency testing and potential immunogenicity resulting in increased numbers of hemophiliacs who form antibodies to factor VIII used as therapy. The potency of these products can be influenced by the state of activation of the factor VIII. This activated state can possibly be detected by the presence of factor VIII fragments known to be formed by thrombin cleavage, by an increase in activity relative to antigen, or by assays relatively specific for activated factor VIII. All factor VIII concentrates available in the U.S. or under review by FDA for licensure are being studied in an attempt to characterize their factor VIII and determine if there are differences among then in activation.

PHS 6040 (Rev. 5/92)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 07012-01-LHT

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Epitope mapping of antibodies to factor VIII

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

William Fricke, MD
Don Lebel, BS
Eleanor Koo, MS

COOPERATING UNITS (if any)

none

LAB/BRANCH

Hemostasis and Thrombosis

SECTION

Division of Hematology

INSTITUTE AND LOCATION

Center for Biologics Evaluation and Research

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.4	.1	.3

CHECK APPROPRIATE BOX(ES)

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

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

Plasma from two patients with antibodies to factor VIII are being studied to identify the epitopes on factor VIII that are recognized by the antibodies. The general approach has been to enzymatically cleave factor VIII and then immunoprecipitate the resulting fragments using patient immunoglobulin covalently coupled to protein A - Sepharose beads. Following digestion of factor VIII by thrombin, fragments of 50 kD (the A1 region - which is further cleaved to 30 kD and 20 kD pieces), 43 kD (the A2 region), and 73 kD (the light chain consisting of fragments A3, C1, and C2) are generated. Patient B's plasma immunoprecipitates the light chain and both the A1 and A2 fragments, whereas patient P's plasma precipitates the light chain only. As a control a monoclonal antibody with known specificity for an epitope in the aminoterminus of the A2 fragment is used. Immunoprecipitation of trypsin cleaved factor VIII has been unsuccessful. It is unclear as to why, but possible explanations include destruction of the epitopes by digestion and insufficient protein to be detected by immunoblotting.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 07013-01 LHT

PERIOD COVERED

October 1, 1992 to September 30, 1992

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

Epitope mapping of antibodies to von Willebrand factor

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

William Fricke, MD
Don Lebel, BS
Eleanor Koo, MS

COOPERATING UNITS (if any)

none

LAB/BRANCH

Hemostasis and Thrombosis

SECTION

Division of Hematology

INSTITUTE AND LOCATION

Center for Biologics Evaluation and Research

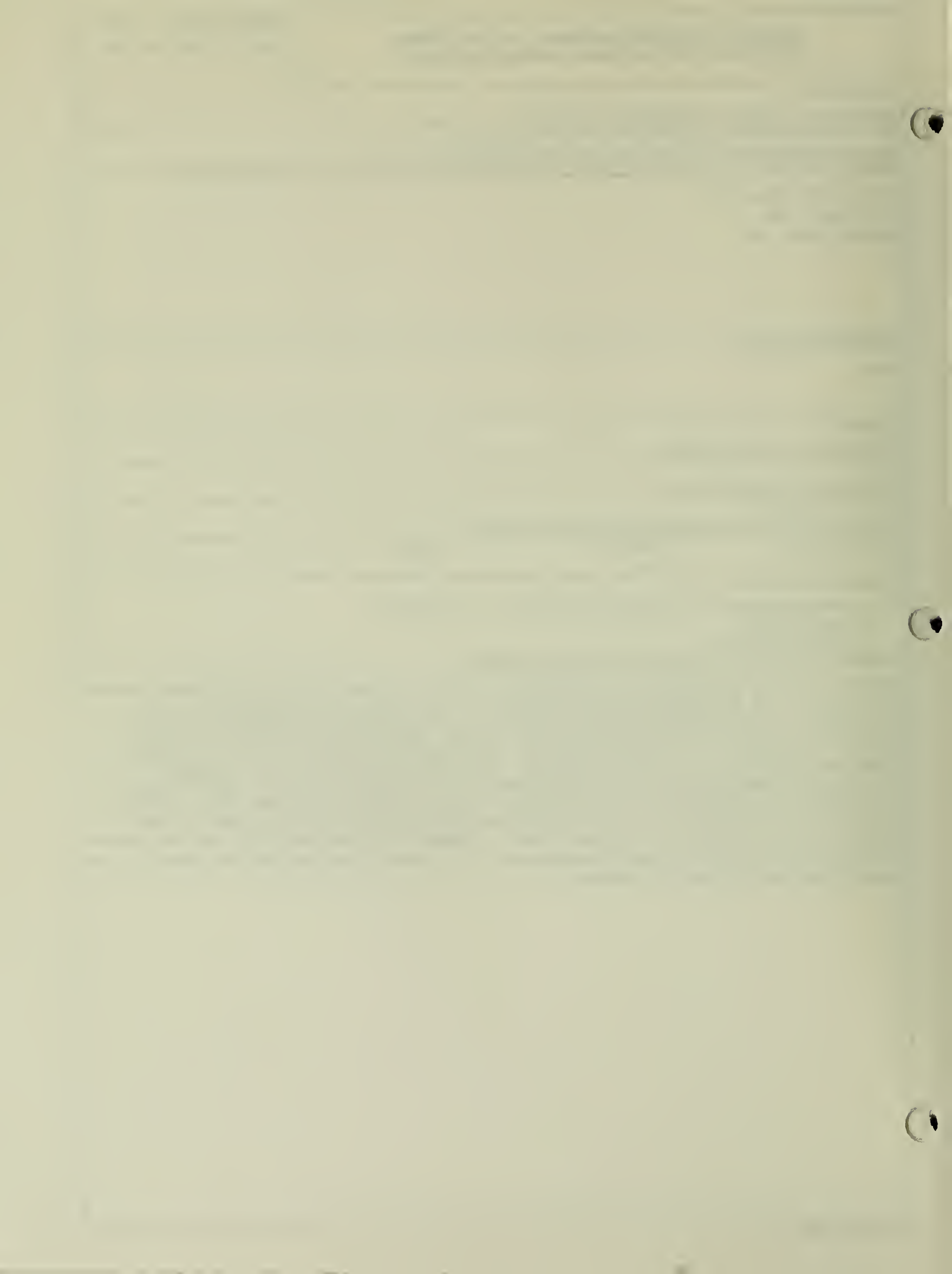
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

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.)

Plasma from two patients with antibodies to von Willebrand factor are being studied to identify the epitopes on von Willebrand factor that are recognized by the antibodies. The general approach has been to enzymatically cleave vWf and then immunoprecipitate the resulting fragments using patient immunoglobulin covalently coupled to protein A - Sepharose beads. Following digestion of vWf by thrombin, numerous fragments ranging in size from about 176 kD to 40 kD are generated. Preliminary experiments using a burro polyclonal antibody have not been sucessful in immunoprecipitating any vWf fragments. It is unclear as to why, but possible explanations include destruction of the epitopes by digestion and insufficient protein to be detected by immunoblotting. Plasma from two patients with antibodies to vWf will be used in these studies when it is clear that the vWf fragments can be immunoprecipitated by this method.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 BH-07009-06-LHT

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
HIV infection due to clotting factor concentrates: Results of the SSP

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

William Fricke, MD
Linda Augustyniak, RN
Dale Lawrence, MD
Alan Brownstein, MSW
Amy Kramer, MPH
Bruce Evatt, MD

COOPERATING UNITS (if any)
National Hemophilia Foundation
Centers for Disease Control

LAB/BRANCH
Hemostasis and Thrombosis

SECTION
Division of Hematology

INSTITUTE AND LOCATION
Center for Biologics Evaluation and Research

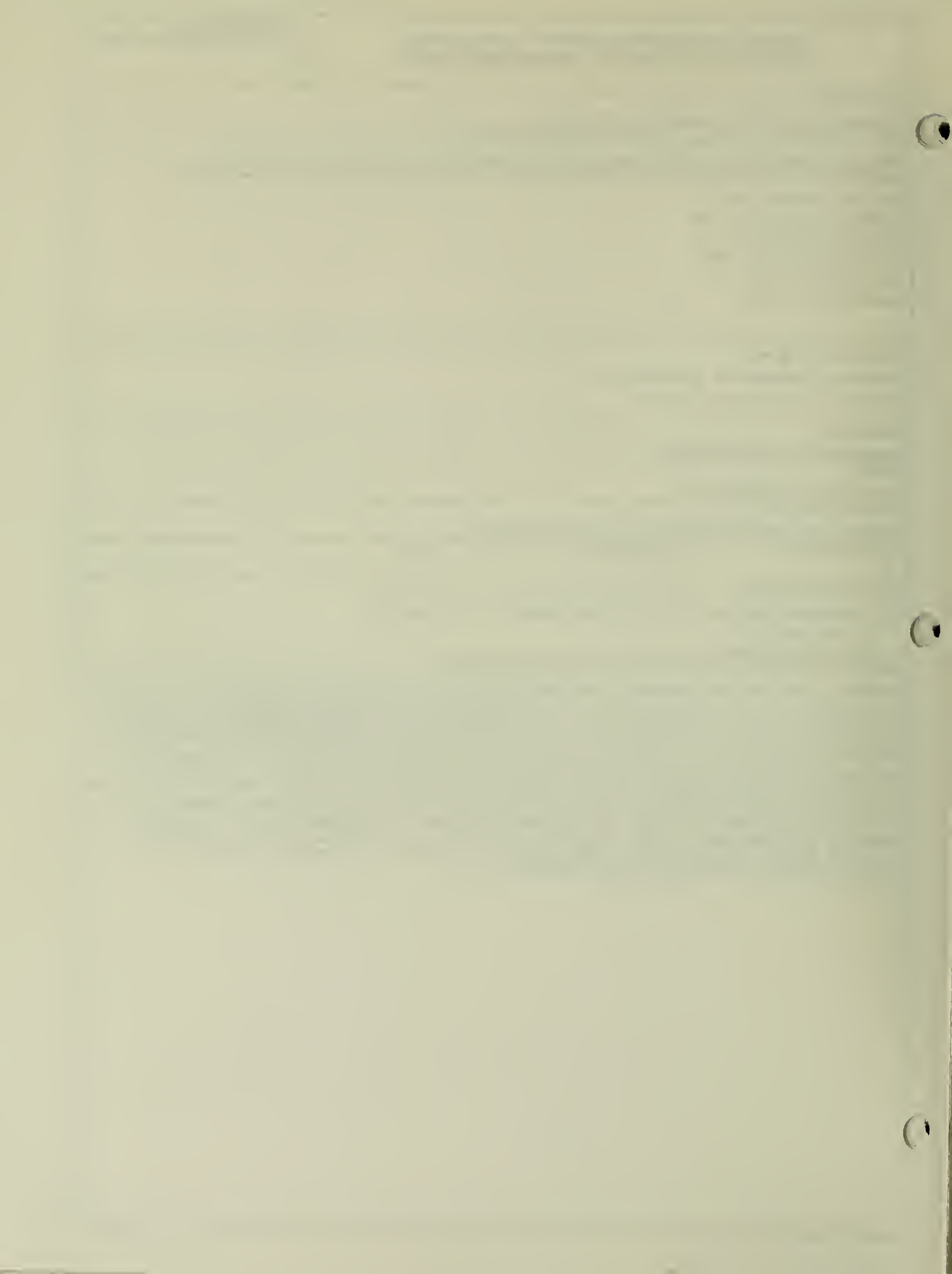
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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.)

Between 1987 and the present, the Seroconversion Surveillance Project has provided the means to monitor the risk of transmission of human immunodeficiency virus (HIV) by clotting factor concentrates. One hundred and thirty-one hemophilia treatment centers in the United States are regularly contacted and data on HIV testing of patients collected. To date, 4366 (46.0%) of 9496 patients have been reported to be seropositive, and thirty-seven new seroconversions identified. Nine of these have met the CDC criteria for seroconversion while taking factor concentrate. None of the nine seroconversions were due to concentrates that had been treated to inactivate viruses and made from plasma that had been tested for HIV antibody. Our results indicate that there is a high prevalence of seropositivity in affected patient groups, but that the risk of HIV infection from currently available clotting factor concentrates is extremely low.

PHS 6040 (Rev. 5/92)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 07014-01-LHT

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Transcriptional Regulation of Urokinase Plasminogen Activator Gene in Metastatis

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

Toby A. Silverman, M.D., Senior Staff Fellow, HemThr, DH, CBER
Laura Wood, M.S., Biologist, HemThr, DH, CBER

COOPERATING UNITS (if any)

Raya Mandler, Ph.D., Postdoctral Fellow, Department of Pathology, USHUHS, Bethesda, MD

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, NIH Building 29, Room 308, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

CHECK APPROPRIATE BOX(ES)

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

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

Metastasis of tumor cells from the primary site of a malignancy accounts for the majority of fatalities in cancer patients. The process of metastasis is complex and involves several steps including invasion and degradation of the extracellular matrix, intravasation, transit through the vasculature, extravasation, and establishment of new tumor foci distant from the primary tumor. These steps probably involve the actions of several proteolytic enzymes which are regulated at several levels temporally during the metastic process. Through its ability to activate plasminogen to plasmin, uPA is among the enzymes involved in the metastic process. We are using a human osteosarcoma model comprised of cell lines showing varying abilities to form tumors and metastasize in athymic nude mice. The gene encoding uPA is single copy in each of the cell lines. By Northern blot hybridization, the nontumorigenic parental cell line HOS expresses low levels of uPA mRNA while two of its transformed deriatives, AD110 and KRIB, express approximately 5-10 times as much uPA mRNA. Preliminary promoter analysis using CAT assay suggest that the 5' flanking region of uPA contains a negative regulatory element active in HOS cells. The same region appears to confer positive promoter activity in AD110 and KRIB cells. Procedures for isolating nuclei for nuclear run on assays have been optimized and nuclear run on assays are currently underway.

Project Number: 3

Project Title: Transcriptional Regulation of Urokinase Plasminogen Activator (uPA) Gene Expression in Metastatic Tumor Cells.

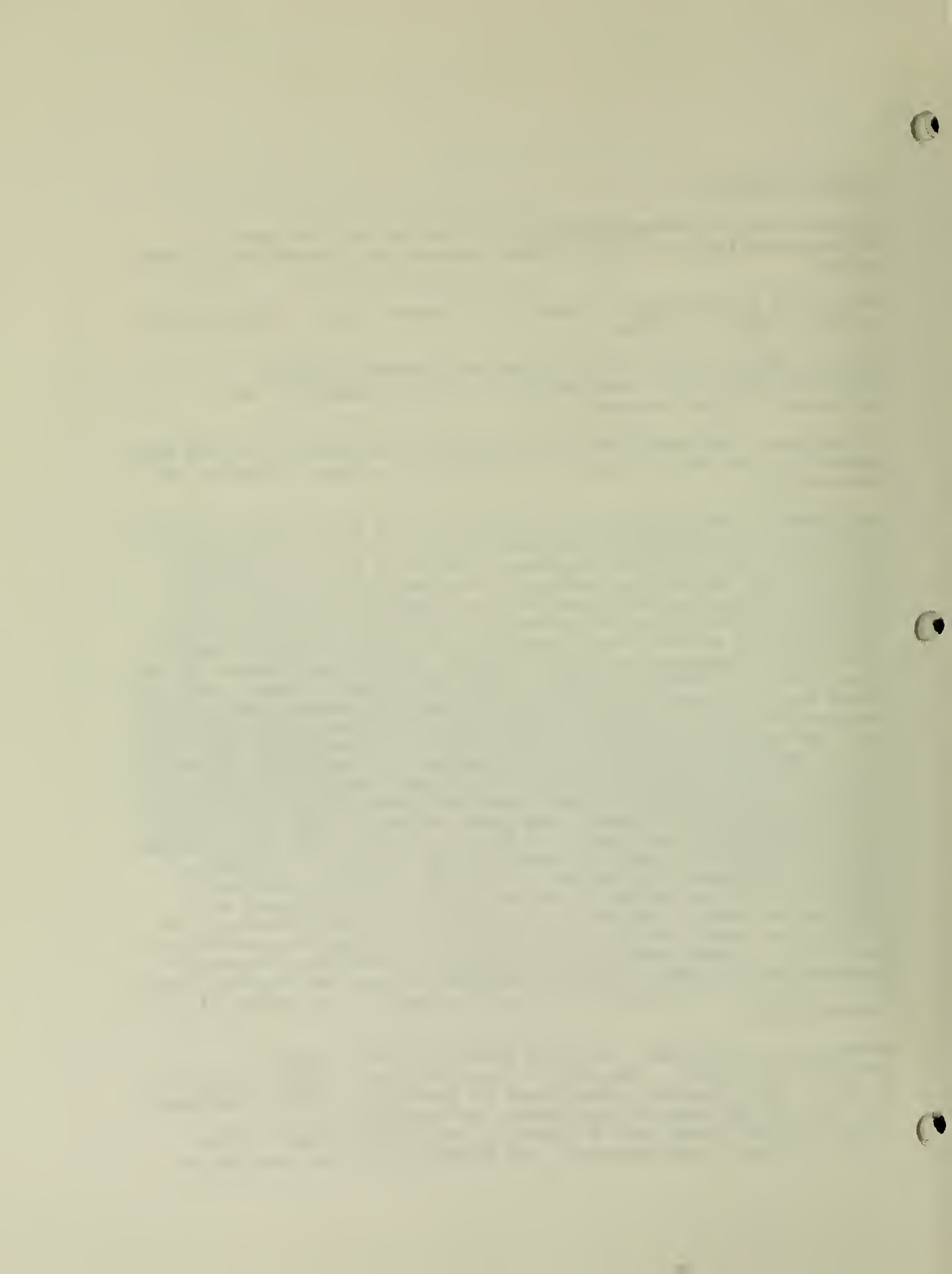
Principle Investigator: Toby A. Silverman, M.D., Senior Staff Fellow, HemThr, DH,CBER

Others: Laura Wood, M.S., Biologist, HemThr,DH,CBER
Raya Mandler, Ph.D., Postdoctoral Fellow, Department of Pathology, USUHS, Bethesda, MD

Objectives: To study the transcriptional regulation of the gene encoding urokinase plasminogen activator in tumor invasion and metastasis.

Background: Metastasis of tumor cells from the primary site of a malignancy accounts for the majority of fatalities in cancer patients. Most current treatment protocols do not adequately eradicate metastasizing cells or secondary tumor foci. The process of metastasis is complex and comprises several steps including invasion and degradation of the extracellular matrix, intravasation either into lymphatics or blood vessels, transit through the vasculature, extravasation, and establishment of new tumor foci in locations distant from the primary tumor. Each of these steps probably involves the actions of several proteolytic enzymes as well as complex interactions of tumor cells with both host defense mechanisms and host matrix. It is likely that each of the proteins involved in this process is regulated temporally during the metastatic process. At present, there is considerable evidence that urokinase plasminogen activator (uPA) is involved in the metastatic process. The best evidence comes from studies of nonmetastatic tumor cells or of transformed cells transfected with an expression vector containing the uPA gene. In these systems, increased UPA expression correlated with enhanced capability of the cells to invade matrigel _in vitro_ and with enhanced metastatic capability _in vivo_. In other studies, UPA activity of tumor cells has been blocked by specific antibodies resulting in fewer metastates _in vivo_. In addition, there is considerable evidence that uPA expression in a variety of tissue culture cells is regulated at least in part at the level of transcription.

Materials and Methods: We have chosen to use a human osteosarcoma model. HOS, AD110, and KRIB cells were obtained from Dr. Raya Mandler, Department of Pathology, USUHS, Bethesda, MD. HOS is an immortalized human osteosarcoma cell line originally obtained from a tumor in the distal femur. These cells are both nontumorogenic and nonmetastatic in nude mice.



KRIB cells were obtained from pulmonary metastases in athymic nude mice injected i.v. with ras transformed HOS cells (KHOS). KHOS cells were obtained from HOS cells after infection with Ki-MuSV. KRIB cells are both tumorgenic & highly metastatic. AD110 cells were obtained from HOS cells transfected with the plasmid pEJras. EJras is an allele of the cellular Ha-ras-1 gene and carries a mutation in codon 12. AD110 cells are tumorigenic but nonmetastatic. Standard molecular biology techniques including transfection, CAT & luciferase reporter gene assays, Northern and Southern blot hybridization, nuclear run on assays and RNase T_1 analysis are being used in this study.

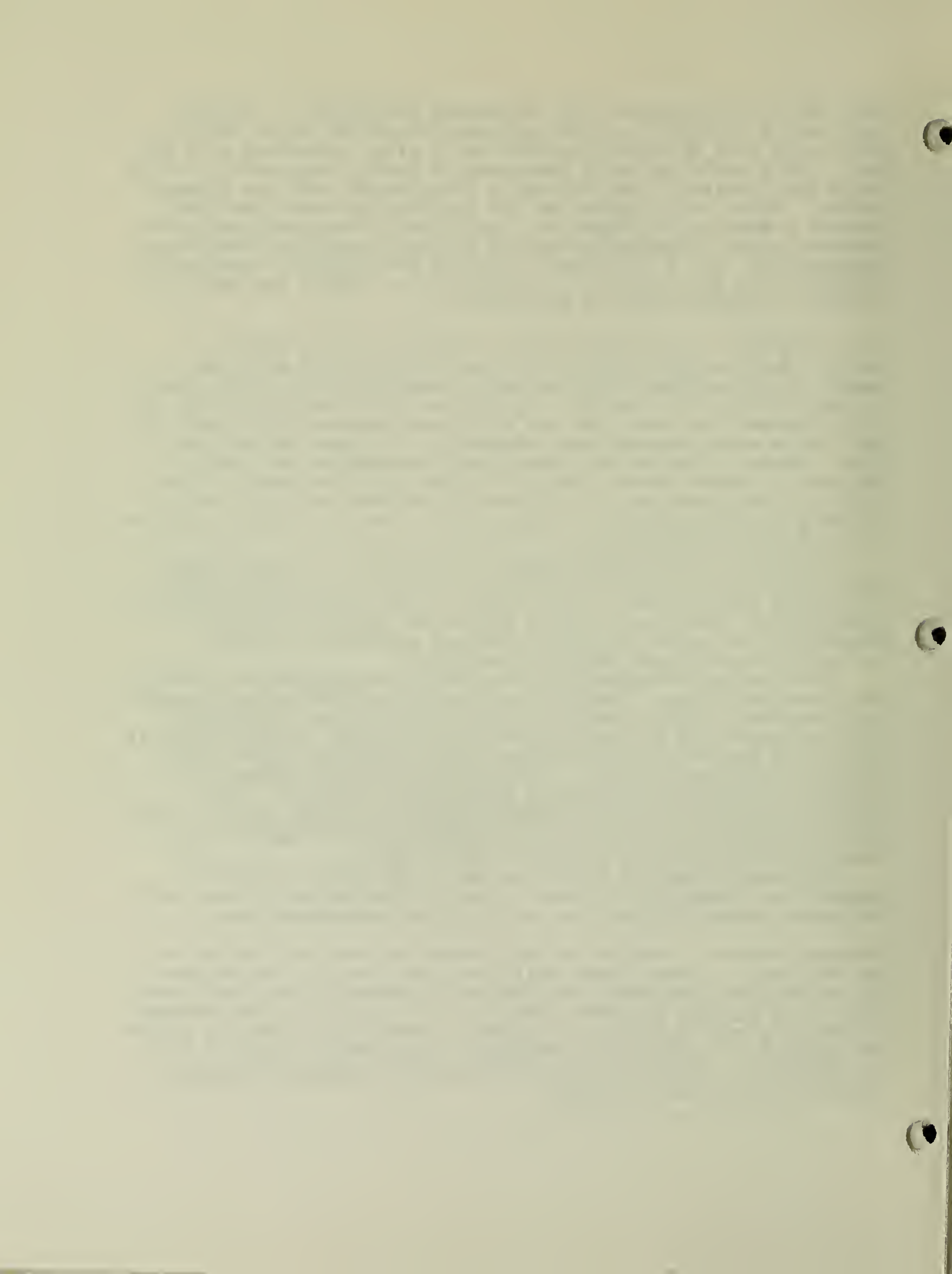
Major Findings: By Southern blot techniques using HEL299, a diploid human embryonic cell line as a control for gene copy number, we have found that the gene encoding uPA is single copy in HOS, AD110, & KRIB cells. By Northern blot hybridization, HOS cells express low levels of uPA mRNA when compared with AD110 & KRIB cells which express approximately 5-10 times as much uPA mRNA. However, preliminary experiments comparing the level of uPA mRNA in these osteoarcoma cell lines with the level of uPA mRNA in a carcinoma cell line known to secrete very high levels of uPA protein show that all three cell lines show relatively low expression of uPA message.

In order to determine whether differences in steady state mRNA levels are due to differences in the rate of transcription of the uPA gene in the 3 cell lines, we have begun nuclear run on experiments. Thus far, we have optimized the conditions for preparation of nuclei and have prepared appropriate probes including positive and negative controls.

We have begun optimizing transfection techniques for each of the three cell lines. Preliminary results of CAT assays suggest that sequences within the first 2400 bases of the 5' flanking sequence behave as a negative regulatory element in HOS cells. In both AD110 and KRIB cells, however, the same 2400 bases confer positive promoter activity. We are still in the process of finding an appropriate internal transfection control. This control will enable us to compare promoter activity from one cell line to another by allowing us to control for differences in transfection efficiency among the cell lines.

We have prepared a probe for use in an RNase T_1 assay to compare the transcription start site for the CAT constructs with the known transcription start site of the endogenous gene.

Proposed Course: Studies of the transcriptional regulation of the uPA gene in these tumor cells will continue if the nuclear run on analysis indicates regulation of expression at this level. Additional in vivo promoter analysis will continue in an attempt to identify both positive and negative regulatory elements in the uPA promoter. In addition, attempts to map these sites in vivo in chromatin by DNase I hypersensitive site mapping & genomic footprinting are anticipated.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 BH 07015-01-LHT

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Plasminogen Activator Inhibitor-1 Gene Expression in Tumor Metastasis

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

Toby A. Silverman M.D., Senior Staff Fellow, HEMTHR, DH, CBER
Laura Wood, M.S., Biologist, HEMTHR, DH CBER
Richard Lewis, Ph.D., Chemist, HEMTHR, DH, CBER
Kay Schneider M.S., Biologist, HEMTHR, DH, CBER

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Hemostasis and Thrombosis, Division of Hematology, CBER, FDA

SECTION

INSTITUTE AND LOCATION

CBER, FDA, NIH Building 29, Room 308, Bethesda

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

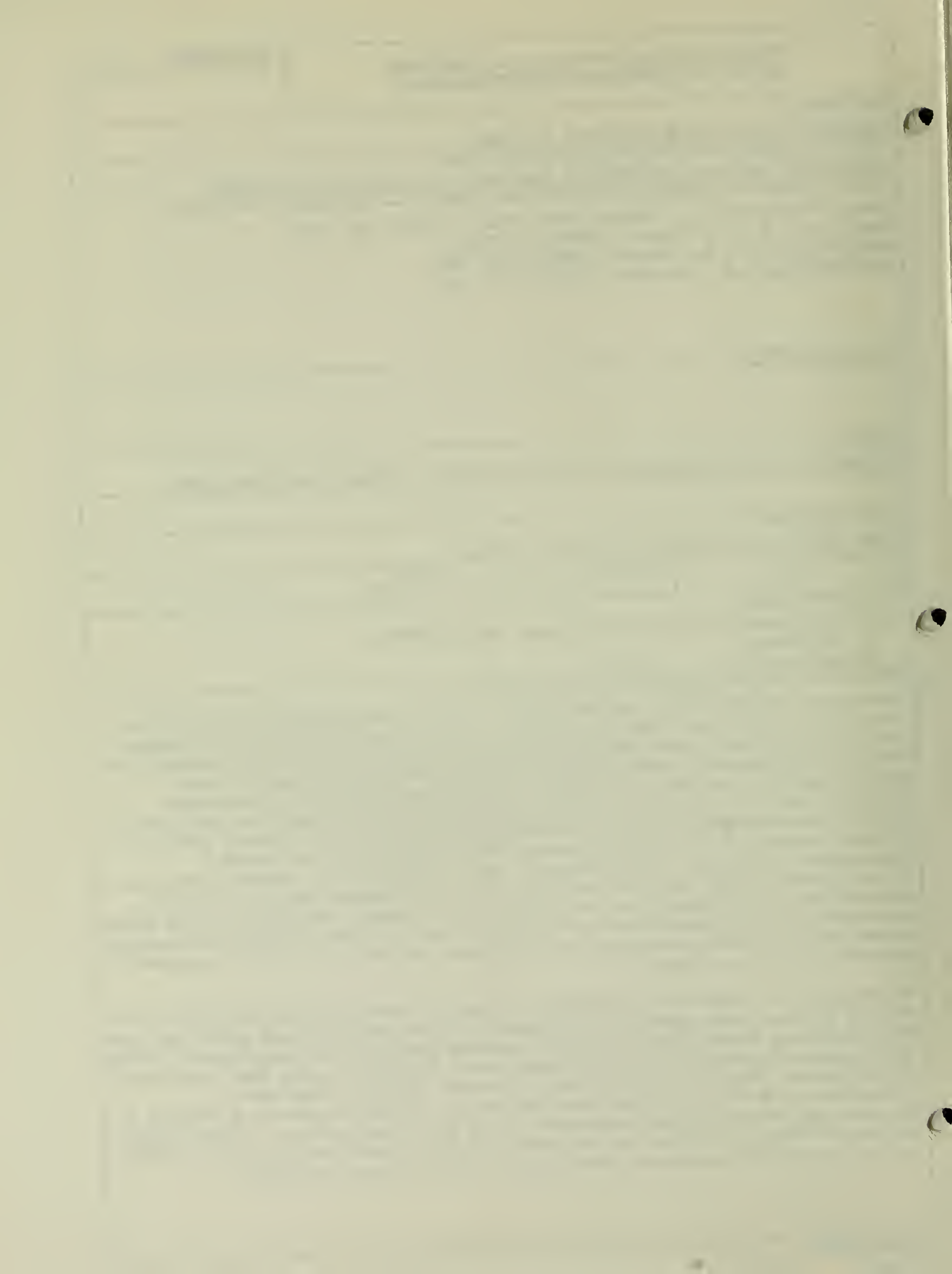
CHECK APPROPRIATE BOX(ES)

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

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

Metastasis of tumor cells from the primary site of a malignancy accounts for the majority of fatalities in cancer patients. The process of metastasis is complex and involves several steps including invasion and degradation of the extracellular matrix, intravasation, transit through the vasculature, extravasation, and establishment of new tumor foci distant from the primary tumor. These steps probably involve the actions of several proteolytic enzymes which are regulated at several levels temporally during the metastatic process. UPA is among pivotal enzymes involved in the metastic process through its ability to activate plasminogen to plasmin. The activity of uPA in tumor cells is probably regulated by the binding of uPA with the inhibitor PAI-1. Preliminary data from a human osteosarcoma model comprised of cell lines showing varying abilities to form tumors and metastasize in athymic nude mice has shown that the level of membrane bound UPA rather than the level of secreted uPA correlates with the invasive and metastatic behavior of the tumor cells.

By Southern blot technique using HEL299, a diploid human embryonic cell line, as a control for gene copy number, we have found that the genes encoding PAI-1, and uPAR are single copy in HOS, AD110, and KRIB cells. By Northern blot hybridization, AD110 cells express approximately 2-3 fold more steady state PAI-1 mRNA than either HOS or KRIB cells. AD110 and KRIB express equivalent levels of uPAR mRNA (approximately 5-10 fold greater than HOS cells). Further studies are underway to evaluate the transcriptional regulation of the gene encoding PA1-1 in these cells. The role of PAI-1 in regulating the binding of uPA to uPAR and the effect of PAI-1 on the invasive and metastatic properities of AD110 will be evaluated.



Project Number 4:

Project Title: Regulation of Plasminogen Activator Inhibitor - 1 Gene Expression in Tumor Metastasis

Principle Investigator: Toby A. Silverman, M.D., Senior Staff Fellow HemThr, DH, CBER

Others: Laura Wood, M.S., Biologist, HemThr, DH,CBER
Richard Lewis Ph.D., Chemist, Hem Thr, DH, CBER
Kay Schneider M.S., Biologist, HemThr, DH, CBER

Objectives: To study the role of PAI-1 in tumor invasion and metastasis and to examine the regulation of PAI-1 gene expression in tumor invasion & metastasis

Background: Metastasis of tumor cells from the primary site of a malignancy accounts for the majority of fatalities in cancer patients. Most current treatment protocols do not adequately eradicate metastasizing cells or secondary tumor foci. The process of metastasis is complex and comprises several steps including invasion and degradation of the extracellular matrix, intravasation either into lymphatics or blood vessels, transit through the vasculature, extravasation, and establishment of new foci in locations distant from the primary tumor. Each of these steps probably involves the actions of several proteolytic enzymes as well as complex interactions of tumor cells with both host defense mechanisms and host matrix. It is likely that each of the proteins involved in this process is regulated temporally during the metastatic process. Through its ability to activate plasminogen to the proteolytic enzyme plasmin, uPA is likely to be among the enzymes involved in the metastatic process. The activity of uPA is regulated at several levels including transcription, as discussed in Project 3. In addition, it is likely that the activity of UPA is regulated by binding of uPA to its specific receptor (uPAR) and by interaction of uPA with the inhibitor PAI-1.

Materials: We have chosen to use a human osteosarcoma model. HOS, AD110, and KRIB cells were obtained from Dr. Raya Mandler, Department of Pathology, USUHS, Bethesda, MD. HOS is an immortalized human osteosarcoma cell line originally obtained from a tumor in the distal femur. These cells are both nontumorgenic and nonmetastatic in athymic nude mice. KRIB cells were obtained from pulmonary metastases in athymic nude mice injected i.v. with ras tranformed HOS cells (KHOS). KHOS cells were obtained by infection of HOS cells with Ki-MuSV. KRIB cells are both are both tumorigenic & highly metastatic. AD110 cells were obtained form HOS cells transfected with the plasmid pEJras.

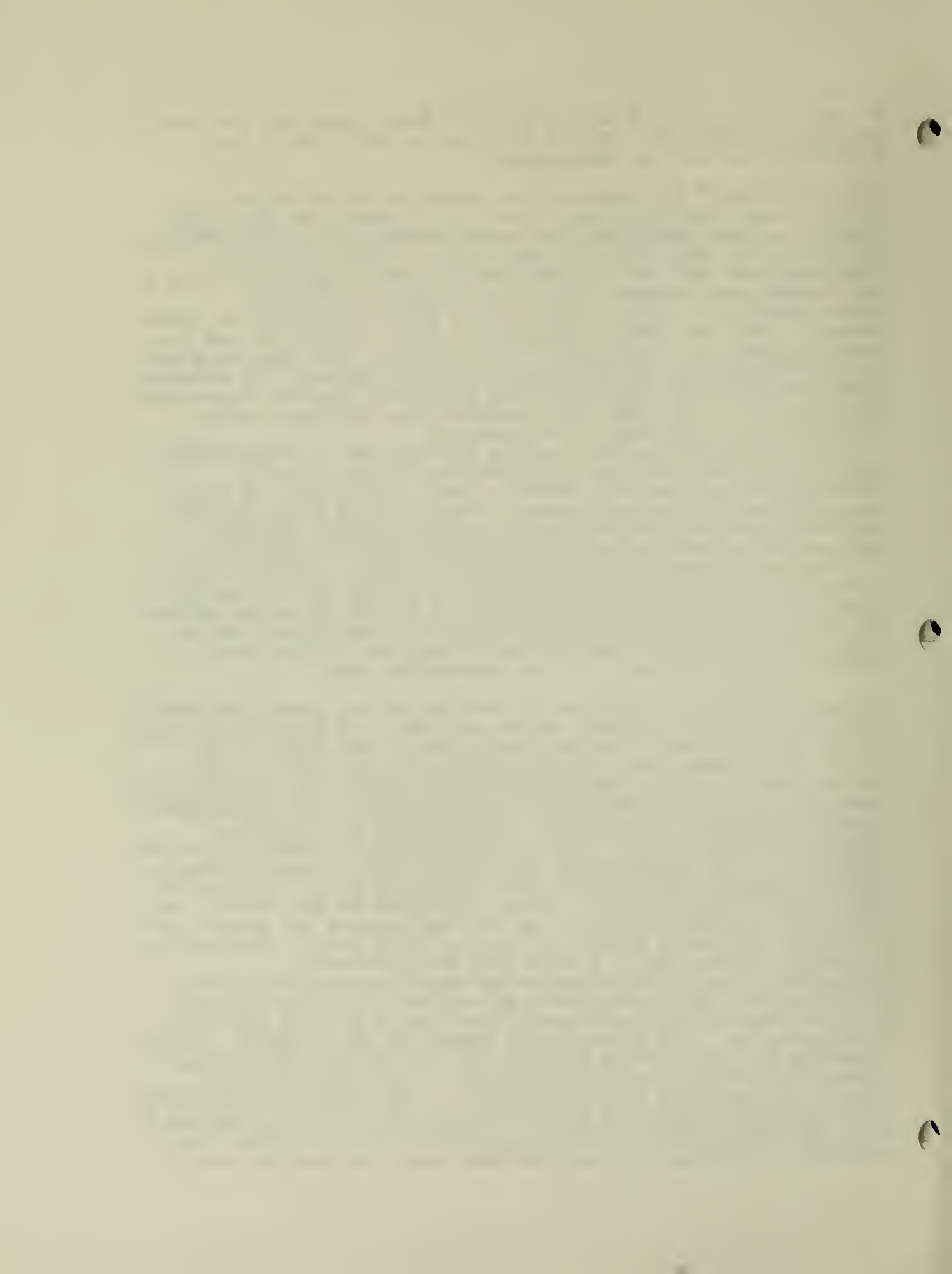
EJ ras is an allele of the cellular Ha-ras-1 gene and carries a mutation in codon 12. Unlike KRIB cells, AD110 cells are tumorgenic but are not metastatic.

Major findings: By Southern blot technique using HEL299, a diploid human embryonic cell line, as a control for gene copy number, we have found that the genes encoding PAI-1 and UPAR are single copy in HOS, AD110, & KRIB cells. By Northern blot hybridization, HOS cells & KRIB cells express very low levels of PA1-1 mRNA when compared with AD110 cells which express approximately 2-3 times as much PAI-1 mRNA. It should be noted, however, that the levels expressed in all 3 cell lines are low, requiring prolonged exposure of the Northern blot despite probe specific activities in the range of 1-2X10^9 cpm/µg. By Northern blot hybroidzation, both AD110 and KRIB cells express equivalent high levels of uPAR mRNA when compared with HOS cells which express very low levels of uPAR message.

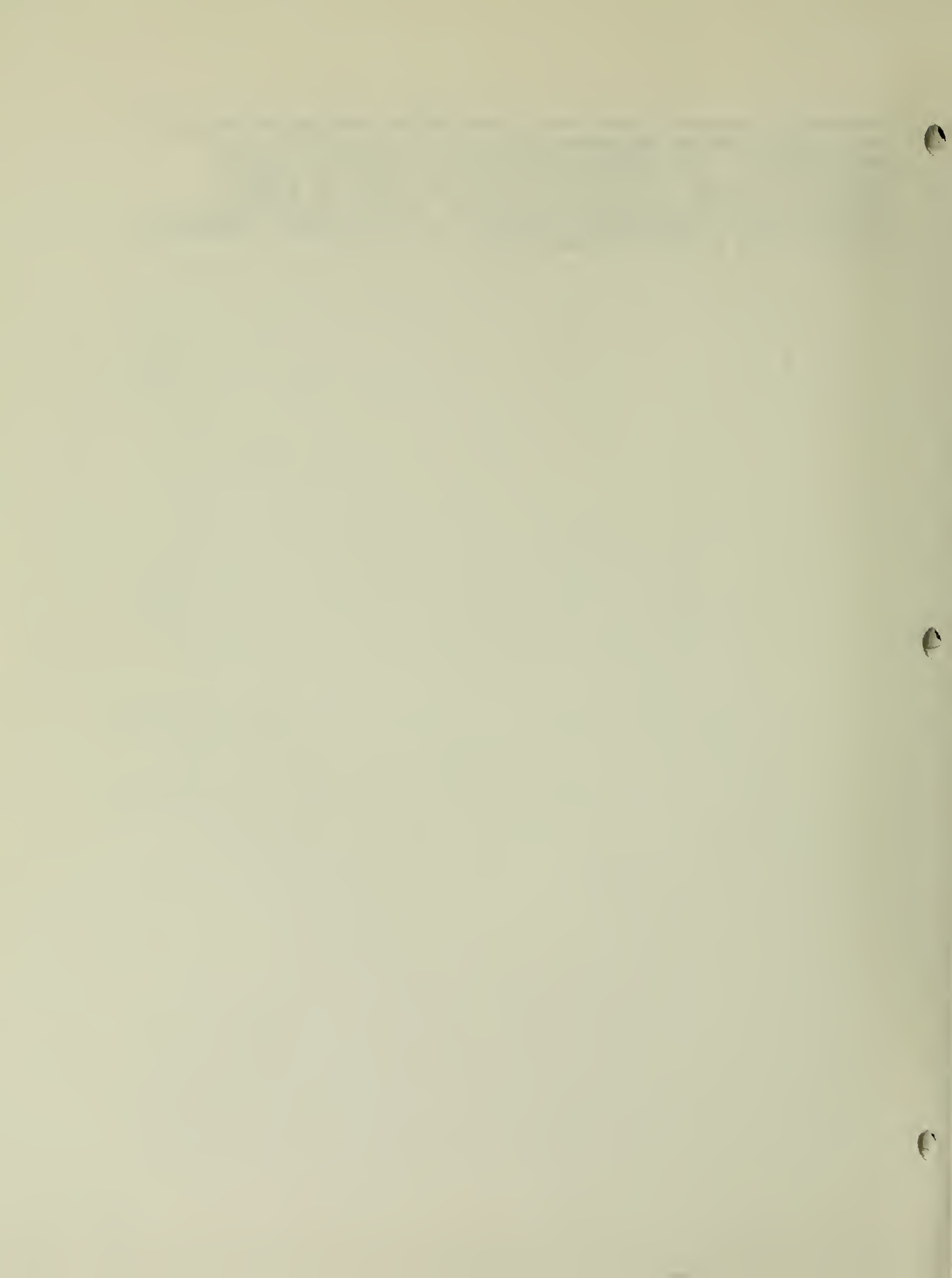
In order to determine whether differences in steady state PAI-1 mRNA levels are due to differences in the role of transcription of the PAI-1 gene in the 3 cell lines, we have begun nuclear run on experiments. Thus far, we have optimized the conditions for preparation of nuclei and have prepared appropriate probes including positive and negative controls.

We have cloned several fragments of the 5′ flanking region of the PAI-1 gene into a CAT vector and are in the process of cloning an appropriate fragment into an riboprobe vector system for use in an RNase T_1 assay to verify that the transcription start site for the CAT constructs corresponds to the known transcription start site for the endogenous gene.

Proposed Course: In addition to looking at the transcriptional regulation of PAI-1 in HOS, AD110, and KRIB, we also plan to look at the effect of PAI-1 protein secretion on the behavior of AD110. Preliminary data indicate that although AD110 cells secrete uPA in levels comparable to KRIB, they show significantly lower levels of membrane bound activitiy. The level of membrane bound uPA appears to correlate with invasive & metastatic behavior in these cells. Thus, it appears that regulation of uPA activity in this osteosarcoma model occurs post translationally. Using antibodies specific for uPA, uPAR, and pAI-1, we plan to look at the distribution of these 3 proteins on HOS, AD110, and KRIB cells by FACS analysis. We hope to measure the global rate of protein synthesis by HOS, AD110, and KRIB cells. The rate of synthesis of uPA, PAI-1, & uPAR will be measured by immunoprecipitation following pulse chase incorporation of ^{35}S methionine and the overall rate of synthesis of these proteins will be compared with the steady state level of mRNA for these three proteins. The steady state number of uPA receptor, percent occupancy by uPA, and the affinity of the receptor for the uPA ligand will be measured by saturation & Scatchard analysis. In order to demonstrate a role for PAI-1 in the biologic behavior of AD110 cells as opposed to KRIB cells, we will look for methods by which to inhibit PAI-1 activity in AD110 cells or enhance its activity in KRIB cells. Such methods could include the use of



saturating amounts of anti PAI-1 antibodies with AD110 cells in an *in vitro* laminin degradation assay. Alternatively, an antisense PAI-1 expression system in AD110 cells or PAI-1 expression system in KRIB cells might be used. By stably transfecting HOS cells with uPA, uPAR, and/or PAI-1, the interactions of these three proteins in the biologic behavior of a single cell line can be addressed.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 BH 07017-01-LHT

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Regulation of Expression of Eukaryotic Initiation Factor-2 alpha

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

Toby A. Silverman M.D., Senior Staff Fellow, HEMTHR, DH, CBER, FDA
Brian Safer, Ph.D., M.D., Head, Section of Protein and RNA Biosynthesis, MHB, NHLBI
Masayuki Noguchi M.D., Section on Protein and RNA Biosynethesis, MHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hemostasis and Thrombosis, DH, CBER, FDA

SECTION

INSTITUTE AND LOCATION

FDA, CBER, NIH Building 29, Room 308, Bethesda, MD

TOTAL STAFF YEARS: 0.1	PROFESSIONAL: 0.1	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.)*

Resting human peripheral blood T cells synethesize proteins at very low rates and contain very low levels of eIF-2 alpha mRNA. During mitogenic activation, the level of eIF-2 alpha mRNA increases at least 50-fold, an effect thought to be due primarily to intranuclear stabilization of the primary transcript. Analysis of sequences (+447 to +457) within the first intron revealed a region with homology to the initiator (Inr) sequence first described by Smale and Baltimore. This Inr element is positioned 450 bases downstreams of the eIF-2 alpha promoter and is oriented to generate an overlapping antisense transcript. Deletion or mutation of the Inr element results in a reproducible 5-8 fold increase in the activity of an eIF-2 alpha promoter driven CAT reporter gene and a corresponding 2.5 fold decrease in activity of an antisense driven luciferase report gene *in vivo* in 293 cells. *In vitro* transcription analysis also reveals antisense transcripts which depend on an intact Inr element and whose 5' ends map to sequences surrounding the Inr consensus sequence. By DNase I footprint analysis and electrophoretic mobility shift assay, we have found a potential cis-regulatory sequence immediately adjacent to the Inr element between +457 and +474. In addition to conferring protection against DNase I, binding of a 43 kD factor also generates hypersensitive sites directly over the Inr element. The antisense orientation of the Inr element with the first intron of the eIF-2 alpha gene suggests a role for the antisense transcript in the regulation of expression of eIF-2 alpha. The role of antisense transcripts and the role of the 43 kD protein in regulation of antisense transcription are currently under investigation.

Project Title: Role of Sequences within the First Intron in the Regulation of Expression of Eukaryotic Initiation Factor-α.

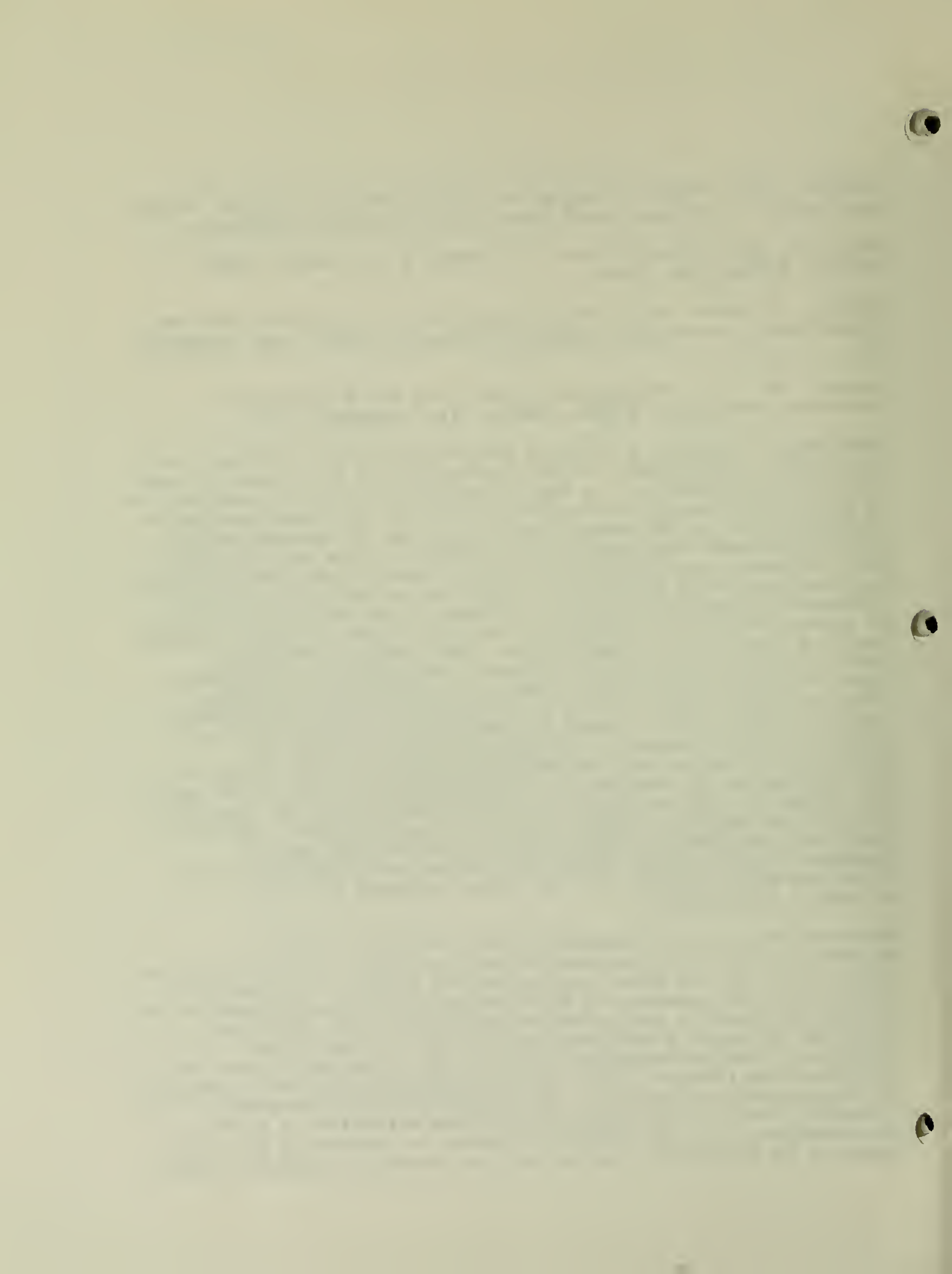
Principle Investigator: T.A. Silverman M.D., Senior Staff Fellow, HEMTHR, DH, CBER

Others: B. Safer Ph.D., M.D. Head, Section on Protein and RNA Biosynthesis, Molecular Hematology Branch, NHLBI, NIH, Bethesda, MD

Noguchi, M., M.D. Section on Protein and RNA Biosynthesis, Molecular Hematology Branch, NHLBI, NIH, Bethesda, MD

Background: Resting G_o T cells are metabolically quiescent and have undetectable levels of DNA synthesis and low levels of RNA and protein production. A rapid increase in protein synthesis is an early event during activation of T cells following antigenic stimulation or by mitogenic lectins such as phytohemaglutinin. Single ribosomes are recruited onto mRNA to form polyribosomes and increased translational activity is seen within the first few hours of mitogenic activation. The rate of protein synthesis increases during the first 24-72 hours of activation and results in levels of protein synthesis that are at least 10-fold greater than the rate in G_o T lymphocytes. This rapid increase in protein synthesis following mitogenic stiumlation of quiescent human T cells occurs at the level of translation initiation. Although elF-2α mRNA is very well translated in both quiescent and activated T cells, human G_o lymphocytes contain very low levels of elF-2α message. During the first 24 hours of activation, however, elF-2α mRNA increases more than 50 fold. Neither changes in the rate of transcription of the elF-2α gene nor changes in the half-life of the message appear to account for all of this rapid and large increase in elF-2α mRNA. We have therefore hypothesized that stabilization of the nuclear precursor sufficient to allow processing and transport to the cytoplasm might account for the large increase seen with mitogenic stimulation.

Previous work in this laboratory has revealed a DNase I hypersensitive site in chromatin mapping to the 5′ portion of the first intron of the gene encoding elF-2α between +220 and +300. Analysis of the sequence of this region revealed an element with perfect homology to the conserved sequence of the Inr first described by Smale & Baltimore as well as two TATA-like sequences. THe Inr sequence (+447 to +457) and one of the TATA-like elements (TACAAT, +353) are oriented opposite the direction of transcription of elF-2α. The second TATA-like element (TACAATAT, +361) is oriented in the same direction as elF-2α. The presence of an Inr element oriented to generate a transcript opposite in direction from the elF-2α transcript suggested that



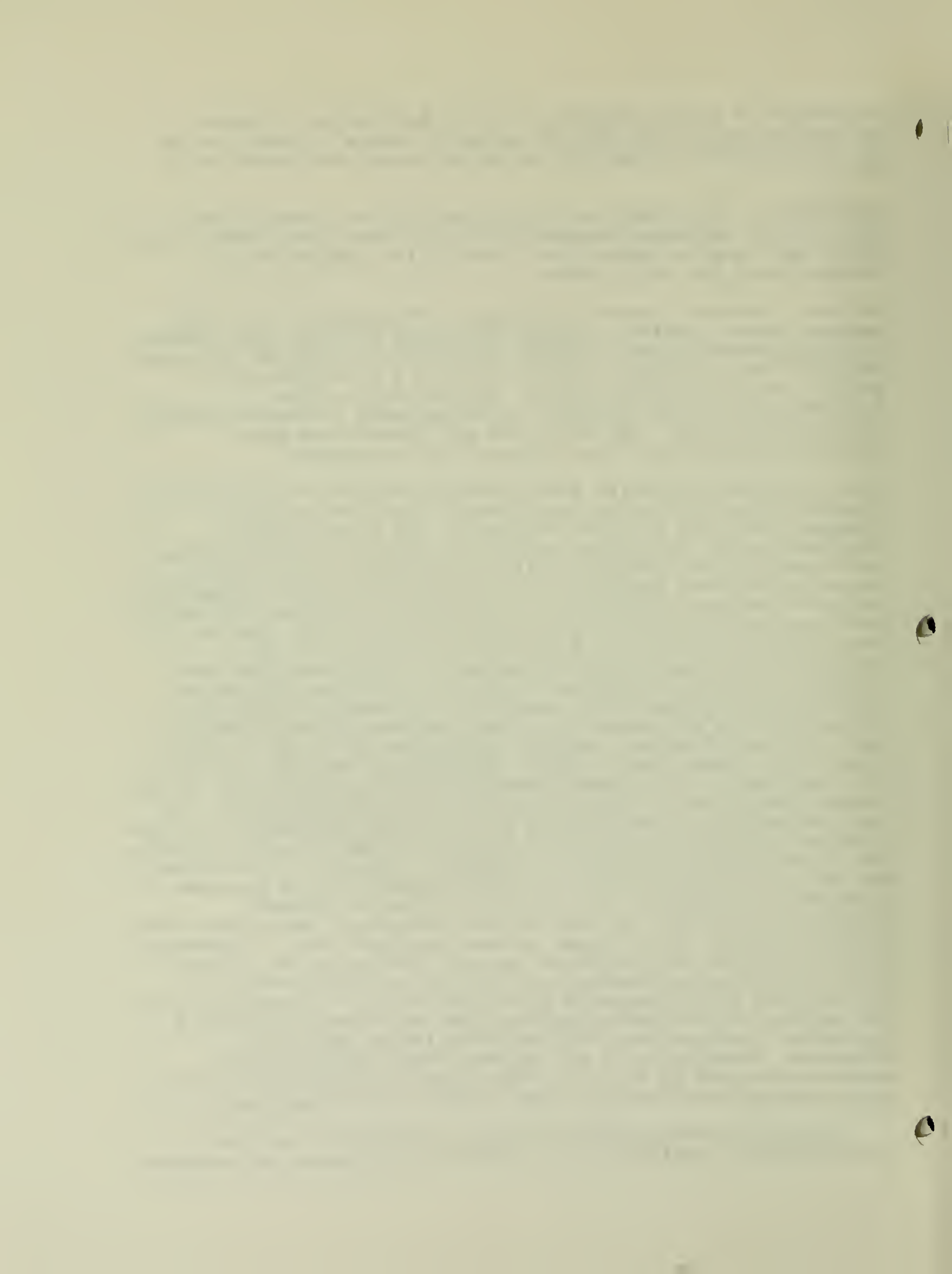
regulation of eIF-2α expression by an overlapping antisense transcript may account for the apparent change in stability of the sense primary transcript during mitogenic activation of T cells.

OBJECTIVES: The objective of this study is to identify and characterize antisense transcripts within the first intron of the eIF-2α gene and to assess their role in the regulation of expression of the eIF-2α gene.

Methods: Standard molecular biology techniques including DNA mediated gene transfer, CAT and luciferase reporter gene assays, <u>in vitro</u> transcription, Northern blot anslysis, primer extension analysis, in vitro DNase I foot printing & electrophoretic mobility shift assay were used. In addition, column fractionation and DNA affinity chromatography & SDS-PAGE protein analysis were used. 293 cells are Ad5 transformed human embryonic kidney cells that express the Ela protein.

Major Findings: We first evaluated the activity of the eIF-2α promoter in a CAT reporter gene assay. This CAT construct extended from -806 to +478 relative to the transcription initiation site. By primer extension analysis we verified that the transcription initiation sites used by the CAT construct mapped to the same region as for the endogenous gene. Removal of 200 bases between +264 and +478 resulted in a reproducible 4-10 fold increase in eIF-2α promoter activity. Because the CAT constructs contain exon 1 & portions of intron 1 in the untranslated leader of the CAT message, we also analyzed the relative promoter activities of both the wild type & deletion constructs by Northern blot analysis. By Northern analysis, a single stranded antisense probe complementary to the first 250 bases of the CAT coding sequence hybridized to an appropriately sized 1.5kb transcript. Removal of sequences between +264 and +478 resulted in a shortened transcript with approximately 5-fold greater hybridization signal than the wild type construct. We next evaluated the contribution of the downstream sequence to the relative strength of the eIF-2α promoter by specifically altering the 4 central bases of the Inr consensus sequence. Alteration of the core 4 bases resulted in an approximately 7-fold increase in relative CAT activity. The mutation of the Inr element reproducibly resulted in greater chloramphenicol conversion than did the deletion of 200 bases between +264 and +478. Alteration of bases outside the consensus sequence but within an in vitro DNase I footprint extending from +459 to +474 resulted in a reproducible 2-fold decrease in CAT activity. We next evaluated the ability of the region spanning the Inr element to drive a luciferase reporter gene when ariented in the antisense direction. Mutation of 3 of the core bases within the Inr sequence resulted in a 2.5 fold decrease in luciferase activitiy when compared with the wild type (+1093 to +179) sequence.

<u>In vitro</u> transcriptional analysis was used to ascertain transcriptional activity of the potential antisense Inr promoter.

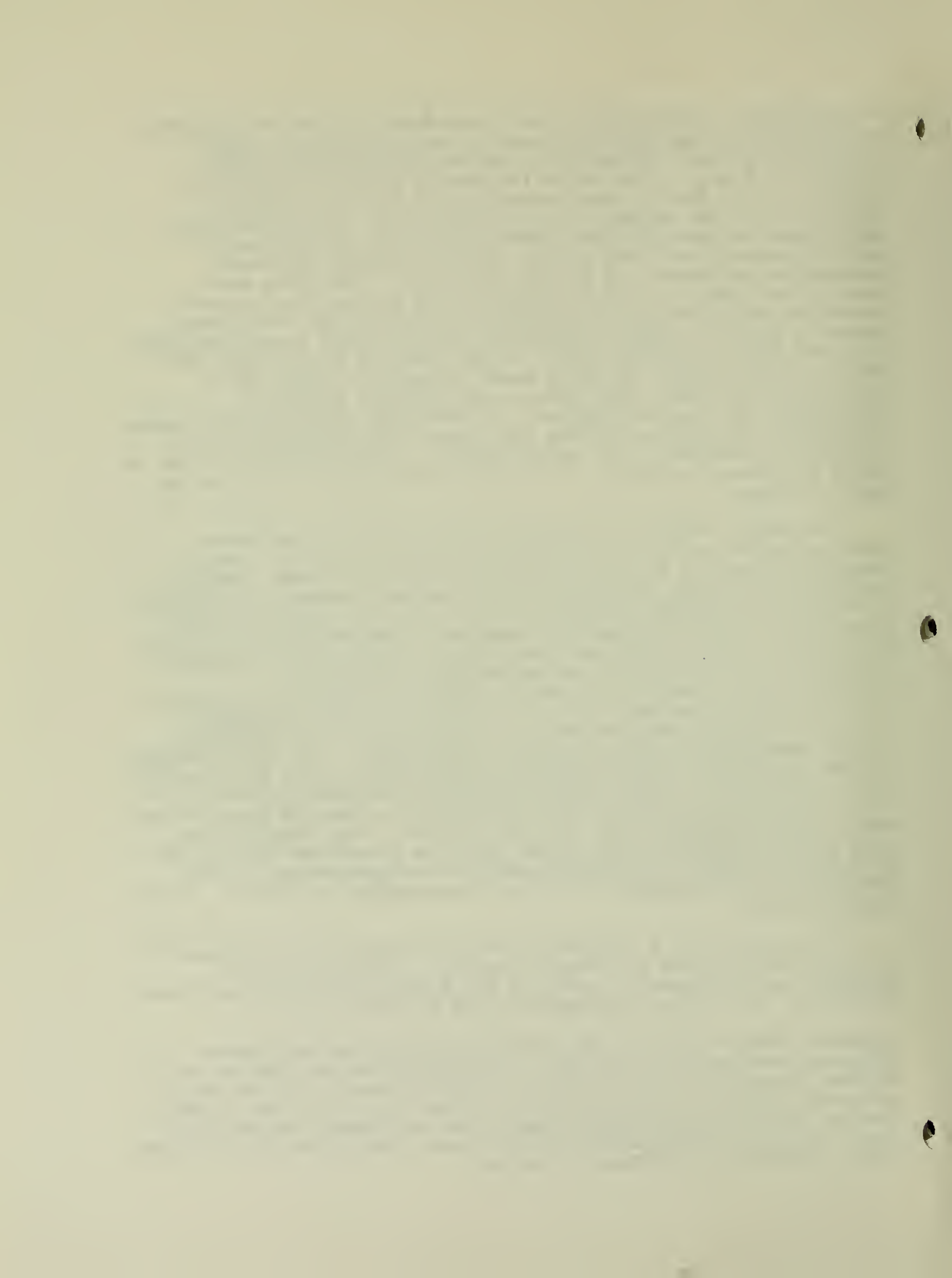


Transcription of the eIF-2α gene truncated at +478 was expected to yield a 478 nucleotide transcript whose abundance might reflect progressive deletion of promoter/enhancer/silencer elements. However, the major transcript from each template varied in size rather than intensity and was shortened in proportion to the extent of the 5′ deletion. The transcript overlapped the exon1/intron1 boundary of elf-2α and was α-amanitin sensitive. The 5′ end of the _in vitro_ antisense transcript was mapped by primer extension. Six sites were identified within a 40 base region and 2 of the start sites mapped to the consensus Inr element. Mutation of the 4 core bases in the Inr element eliminated the _in vitro_ transcript seen with the wild type construct. In contrast to the _in vivo_ CAT data, we were unable to see a sense transcript under the conditions used here even though the _in vitro_ antisense transcript had been eliminated. In addition, mutation of 3 bases immediately downstream of the Inr element (within the DNase 1 footprint) resulted in an approximately 2-fold decrease in the _in vitro_ antisense transcript, a result which differs from our _in vivo_ CAT data.

To detect binding of potential trans-acting regulatory factors in the region, electrophoretic mobility shift assay (EMSA) and DNase I footprint analysis were performed. Using a radiolabelled oligo spanning the Inr region, incubation with the 0.3-0.5M KCl phosphocellulose fraction of K562 nuclear extract generated a single DNA-protein complex. The addition of 25-250 fold molar excess unlabeled specific oligonucleotide eliminated the specific DNA-protein complex whereas nonspecifc oligonucleotide competitors had no effect. The nuclear extract fraction eluting between 0.3 & 0.5 M KCl was found to confer strong protection against DNase I digestion on the noncoding strand immediately adjacent to the Inr element. On the noncoding strand, protection against DNase I digestion extends from +457 to +474 while on the coding stand, protection extends from +451 to +476. In addition, DNase I hypersensitive sites are observed on both strands directly over the Inr element. The footprint as well as the hypersensitive sites could be completed by a 50-250 fold molar excess of an oligonucleotide corresponding to the footprint region whereas an oligo corresponding to the Inr region had no effect.

To identify in a preliminary way the protein(s) responsible for the EMSA & DNase I footprint results, a Hager-Burgess analysis was performed. Specific DNA binding activity was found in protein gel slices corresponding to M_r 40-45,000.

Proposed Course: During the next year using the luciferase reporter gene assay, we propose to further evaluate the roles of the Inr element and the DNase I footprint element in the overall promoter activity of the antisense transcript. Although in the past we have had difficulty mapping the antisense transcript _in vivo_, we hope to demonstrate its presence conclusively by nuclear run on assays and by RNase T_1 assays.



Papers resulting from this project:

1. Silverman, T.A., Noguchi, M., and B. Safer. Role of Sequences within the First Intron in the Regulation of Expression of Eukaryotic Invitation Factor 2α. J. Biol. Chem 267:9738-9742m 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 07018-01-LHT

PERIOD COVERED

Octobe 1, 1991 through September 30, 1992

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

Histopathology and Toxicity of Pertussis Heat-Labile Toxin (PEHLT)

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

Toby A. Silverman M.D., Senior Staff Fellow, HEMTHR, DH, FDA, CBER
Ronald Sekura Ph.D. DAIDS/NIAD, NIH
Yan-Ling Zhang Ph.D. UNIVAX Biologics, Inc.

COOPERATING UNITS (if any)

Russell Brynes M.D., Department of Clinical Pathology, City of Hope National Medical Center, Durarte, CA 91010

LAB/BRANCH

Hemostasis and Thrombosis, Division of Hematology

SECTION

INSTITUTE AND LOCATION

CBER, FDA Building 29, Room 308, Bethesda, MD 20892

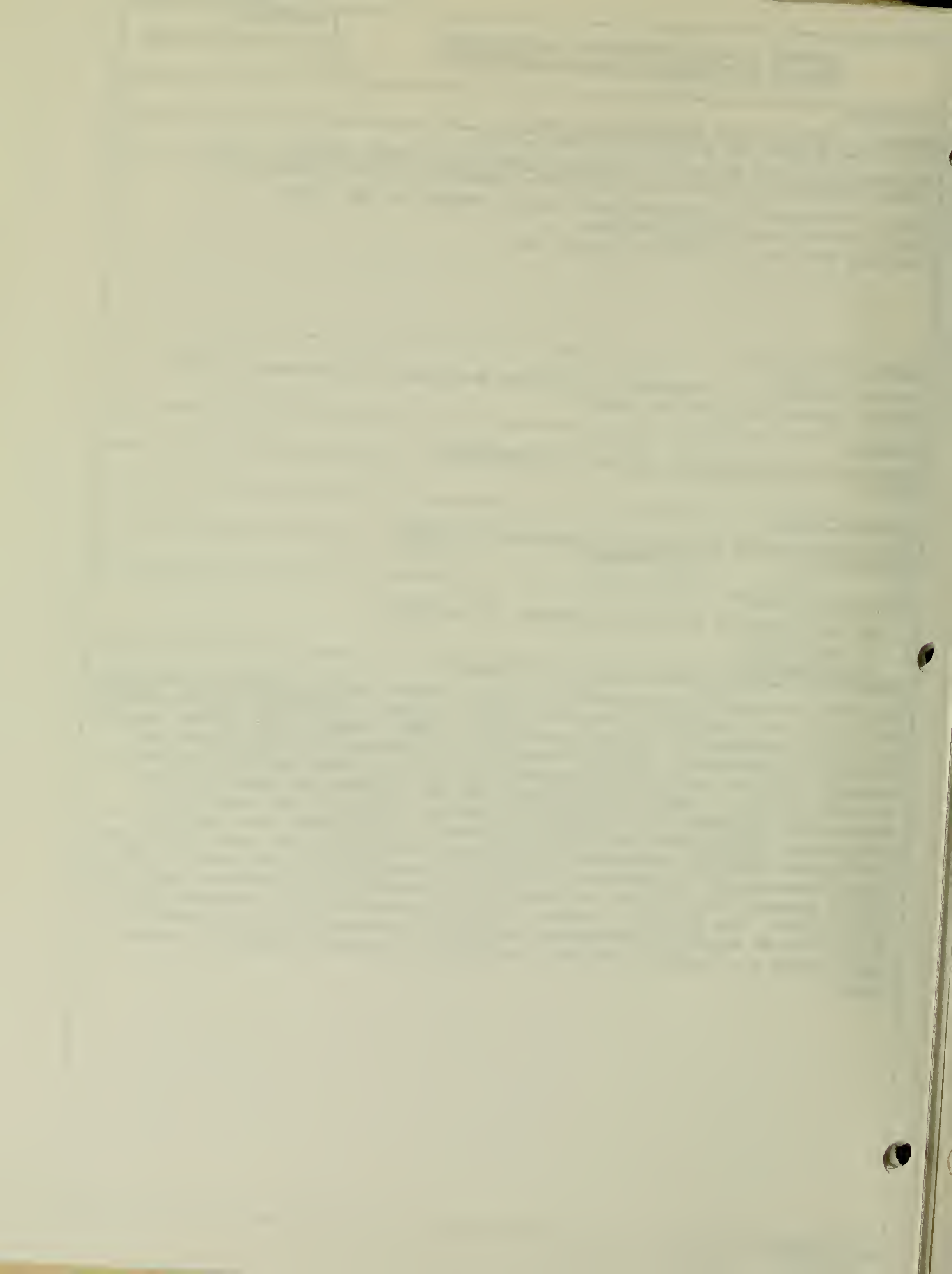
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	—

CHECK APPROPRIATE BOX(ES)

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

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

Pertussis heat-labile toxin (PEHLT) is one of several toxins produced by Bordetalla pertussis. The biochemical and pharmacologic effects of PHELT and its relationship to clinical pertussis in humans is not known. PEHLT exhibits toxic activities when injected subcutaneously, intraperitoneally, or intravenously into rodents. We examined the gross and histophathologic features of a highly purified preparation of PHELT in mice. Highly purified PEHLT (200 pg) injected subcutaneously into newborn mice elicited an early acute inflammatory resonse in the superficial subcutaneous tissues and lower dermis followed by a mononuclear cell perivascular infliltrate, vascular congestion, dermal edema, extravasation of erythrocytes, and finally hemorrhage. Injection of the toxin intraperitoneally into 18-20 gm NIH general purpose mice resulted in thymic involution and a dose dependent decrease in splenic size. The changes in splenic size correlated with the histologic findings of necrosis of erythroid and myeloid precursors in the red pulp. At sublethal doses, PHELT also caused depletion of erythroid and myeloid elements in the bone marrow.



Individual Intramural Project Report

October 1, 1991 through September 30, 1992

Project Title: Pertussis Heat-Labile Toxin (PEHLT): Histopathology and Toxicity to Hematopoietic Elements.

Principle Investigator: Toby A. Silverman M.D., Senior Staff Fellow, HEMTHR, DH, CBER, FDA

Others: R. Sekura, Ph.D., DAIDS/NIAID, NIH
Y-L. Zhang, Ph.D., UNIVAX Biologics, Inc.

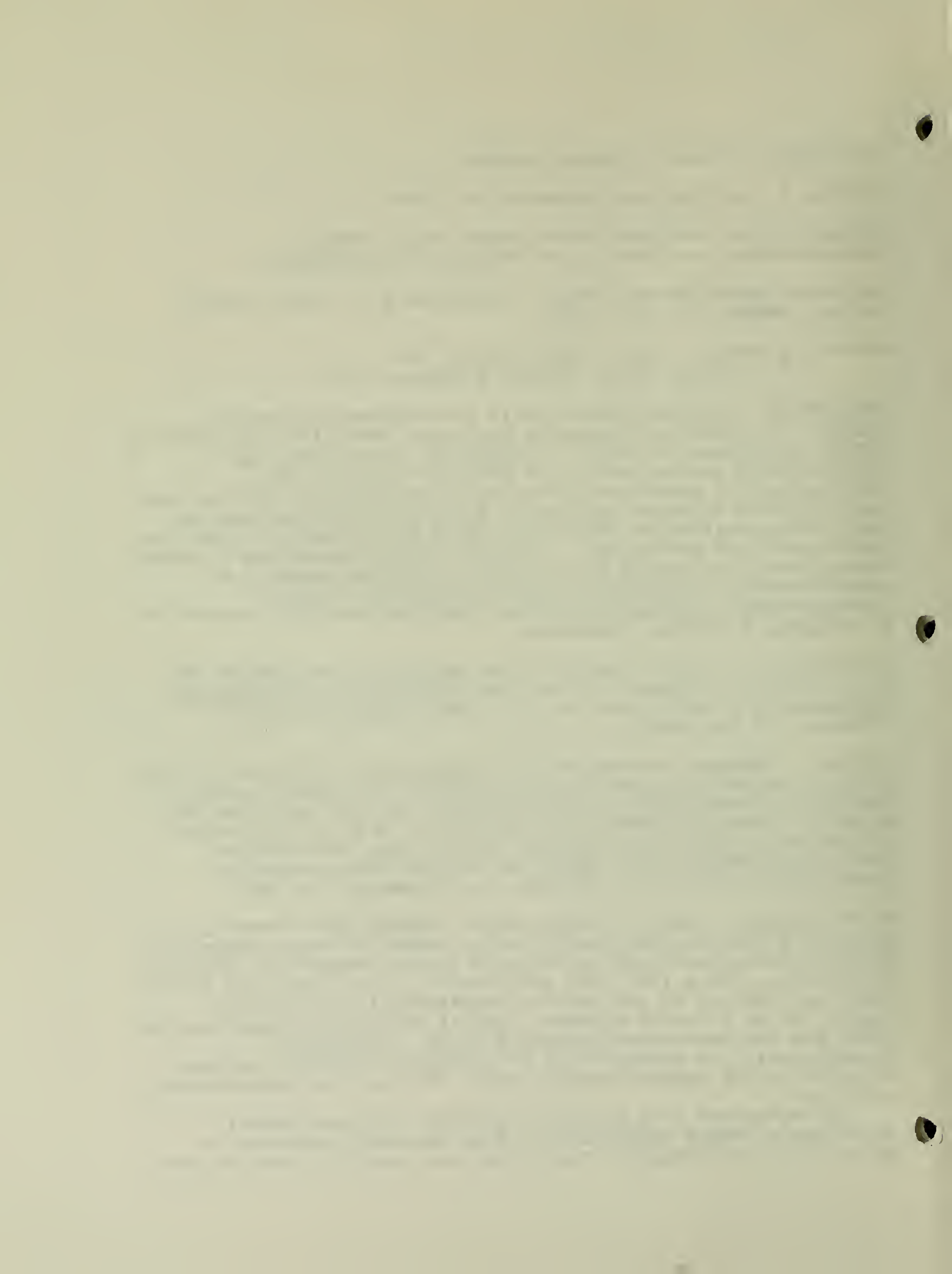
Background: Pertussis heat-labile or dermonecrotic toxin (PEHLT), produced by Bordetella pertussis, was first described by Bordet and Gengou in 1909. Although the biochemical and pharmacologic mode of action of PEHLT is unknown, the idenfication of functionally and/or immunologically similar heat-labile toxins in other pathogenic Bordetella species suggests that toxins related to PEHLT may play a significant role in the pathogenesis of pertussis. Although crude preparations of PEHLT showed toxic activities when injected intraperitoneally or subcutaneously into rodents, few descriptions of the histopathologic changes associated with the heat-labile toxin of B pertussis have been described.

Objectives: The basic goal of this project is to examine the histopathologic effects of the heat labile toxin of Bordetella pertussis as a preliminary step in identifying the target tissue(s) of the toxin.

Methods: Standard histopathologic methods were used during this project including preparation and fixation of tissues, paraffin embedding & hematoxylin & eosin staining of thin sections. For studies of the histopathologic effects in 18-20 gm NIH general purpose mice, the toxin was used as purified. For studies involving neonatal mice, PEHLT was diluted into a solution containing 1 mM DTT, 0.1 mM EDTA, and 2% gelatin in PBS.

Major findings: We (Y-L Zhang and R. Sekura) have recently purified pertussis heat-labile toxin (PEHLT) to near homogeneity. The procedure, performed in the cold in the presence of protease inhibitors, gave a 1350 fold purification. The resulting single chain polypeptide of 140 kDa was completely inactivated by heating at 56 C for 60 minutes. Rabbit antisera prepared against PEHLT did not react with pertussis toxin, filamentous hemagglutinin, or preparations of pertussis adenylate cyclase. In 18-20 gm NIH general purpose mice, the LD_{50} was approximately $11X10^{-9}$gm.

In agreement with previous findings, intraperitoneal injection of PEHLT resulted in a dose dependent decrease in splenic size and weight. The effect was specific since E. Coli



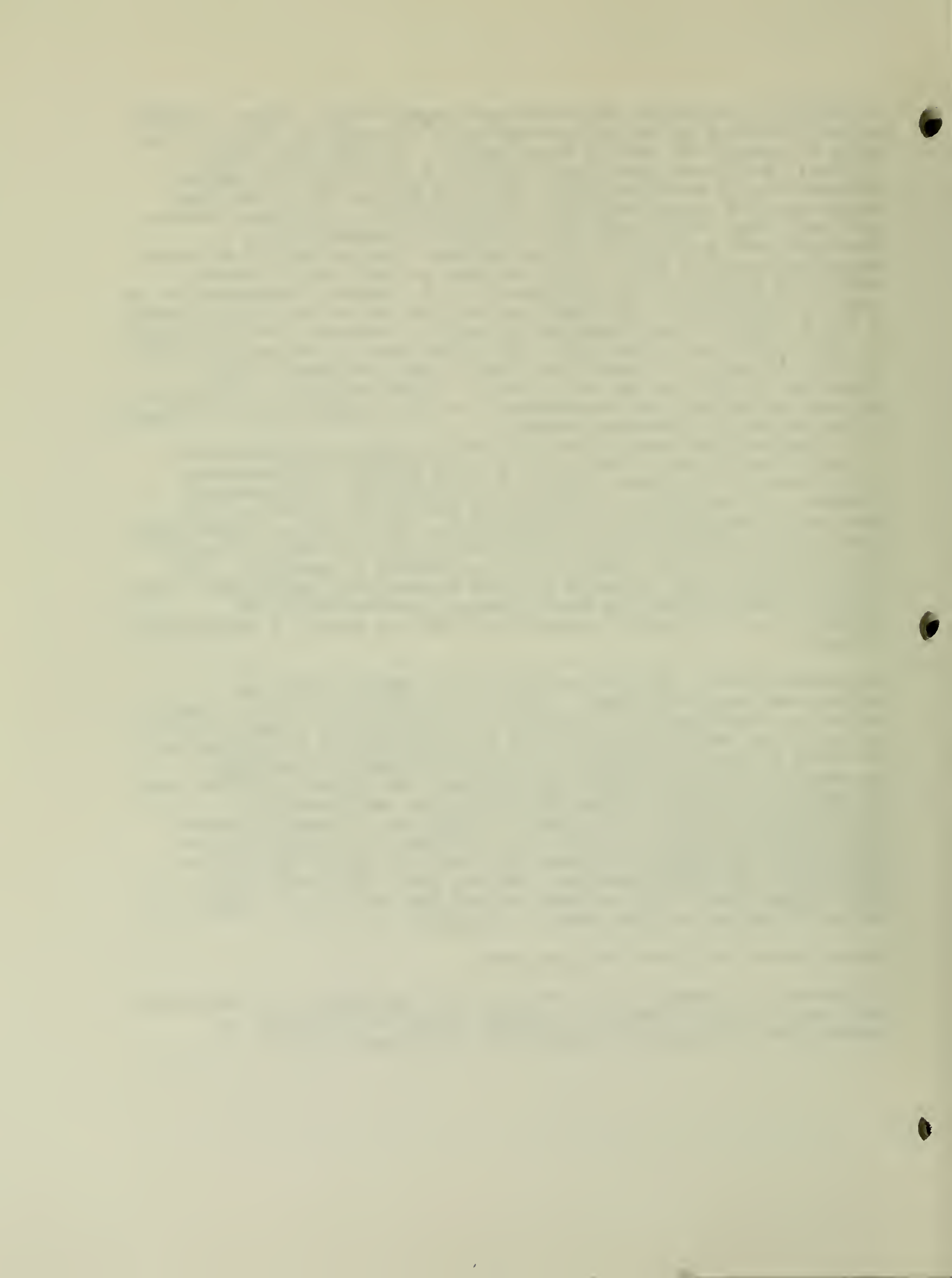
endotoxin & pertussis toxin did not produce this effect. PEHLT caused necrosis of early hemotopoietic elements in both the red pulp of the spleen and in bone marrow. At sublethal does (3.3ng), erythroid precursors in both bone marrow and spleen appeared to be preferentially affected by the toxin although early myeloid precursors were also depleted. At higher doeses (16.5ng), the toxin resulted in subtotal necrosis of all hematopoietic precursors in the spleen. The effect of a single sublethal dose of toxin could be seen as early as 12 hours following injection and persisted for 5-7 days. Regeneration of both the bone marrow and the red pulp of the spleen began between 5 and 7 days and was complete by day 14. Recovery of the weight of the spleen correlated with the appearance of regenerative foci in the red pulp. In addition, PEHLT also producted a dose dependent involution of the thymus. These changes in thymus and splenic white pulp are reminiscent of the changes seen in animals following opiate induced stress.

The earliest change seen in skin following subcutaneous injection of 200pg of PEHLT was a mild diffuse inflammatory infiltrate in the lower dermis and superficial subcutaneous tissues. Although the inflammatory infiltrate at this time consisted predominantly of neutrophils, by six hours, there were also small numbers of mononuclear cells tightly cuffing small blood vessels at the junction of the dermis & subcutis. In addition, the small vessels in this region were congested. These changes were followed by dermal edema, extravasation of erythrocytes, & finally hemorrhage into the dermis & subcutaneous tissues.

Proposed Course: The target cell(s) of PEHLT cannot be determined from this study. The toxin appears to have a specific effect on hematopoietic precursors in the spleen & bone marrow. In skin, the early appearance of an inflammatory infiltrate at the site of injection prior to the development of vascular congestion and hemorrhage suggests that the effect of PEHLT may be due to the local release of vasoactive mediators by inflammatory cells at the site of injection. Based on these observations we hope to look for a suitable in vitro culture system for hematopoietic precursor cells in order to test our hypothesis that PEHLT may be a direct toxin for these cells. If this is the case, then we hope to use the toxin as a ligand for the purification of the specific receptor.

Papers resulting from this project:

Silverman, T., Zhang, Y-L., Brynes, R., and Sekura, R. Pertussis Heat-Labile Toxin (PEHLT): Histopathology and Toxicity to Hematopoietic Elements. (manuscript in preparation).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH-07005-02-LHT

PERIOD COVERED

August 1991-August 1992

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

Identification of Monoclonal Antibodies to Tissue Factor Pathway Inhibitor

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

Richard M. Lewis, PhD, Chemist,
Mary Catherine J. Schneider, MS, Biologist

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hemostasis and Thrombosis Laboratory

SECTION

INSTITUTE AND LOCATION

CBER, Building 29

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

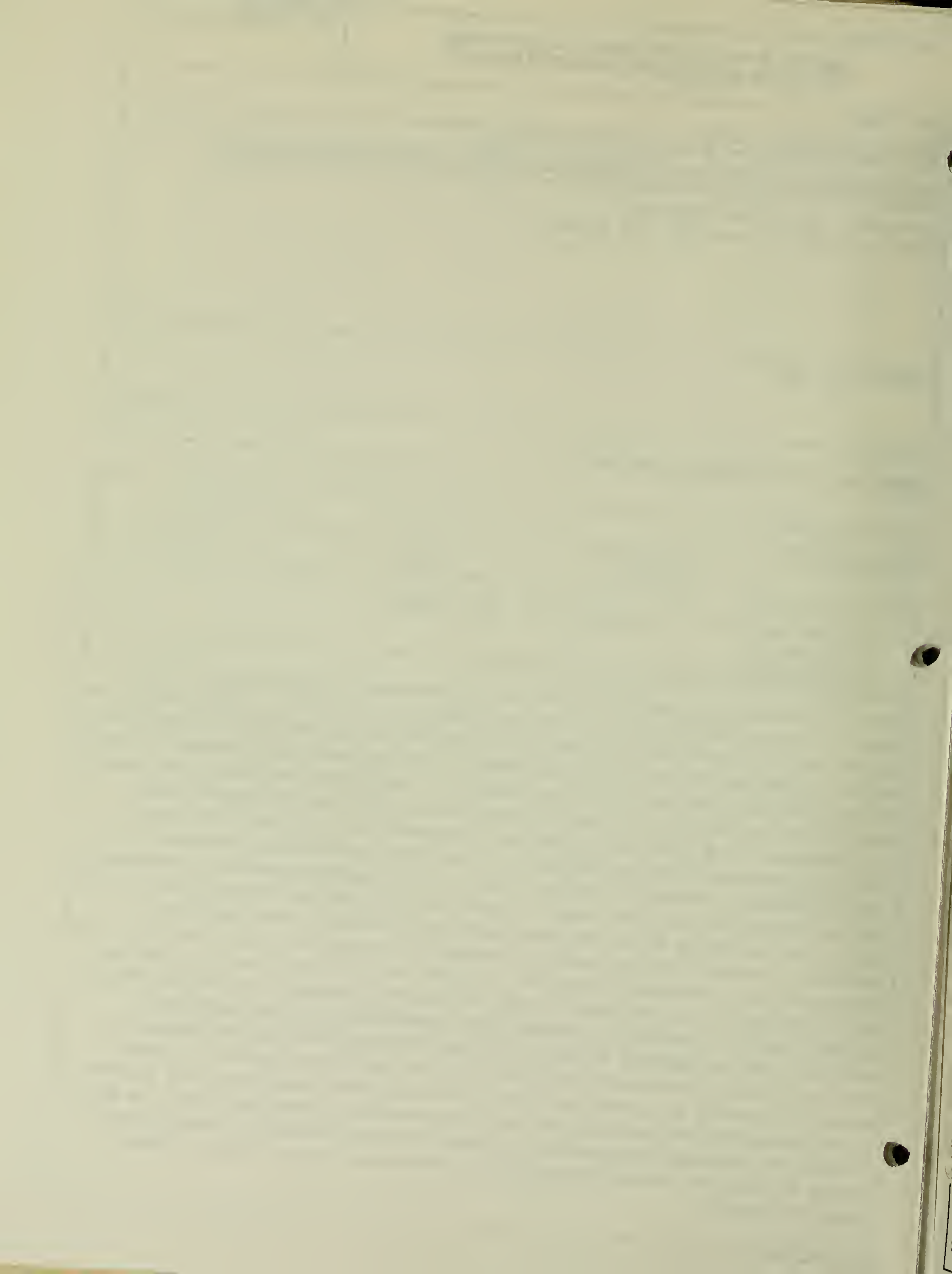
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

Tissue factor pathway inhibitor (TFPI) is a circulating plasma component which binds to coagulation Factor Xa (F Xa) and inhibits F Xa activity. This bimolecular complex can then bind to the Factor VIIa-tissue factor complex (VIIa-TF) and, in so doing, inhibit the Factor X (F X) and Factor IX (F IX) -activating ability of the VIIa-TF. Because TFPI exhibits many functions, a monoclonal antibody which blocked the activity of any specific function would be a useful tool for characterizing this molecule. For this reason mice were immunized with purified TFPI, splenic cells isolated and fused with myeloma cells and the resulting hybrid populations were screened for anti-TFPI activity. In order to identify monoclonal antibodies against the various specific regions of TFPI, a number of assays were developed and used in the screening of the isolated monoclonal hybridomas. One assay was developed to detect antibody which neutralized the inhibitory ability of TFPI for F Xa cleavage of a synthetic substrate. A second activity assay was developed to detect the ability of antibody to block the VIIa-TF inhibitory activity of the F Xa-TFPI complex. Because an antibody which neutralized the F Xa inhibitory activity would be expected to function in the second type of neutralization as well, both assays were necessary to characterize antibody activity. A third antibody screening assay was also used. This technique incorporated the standard ELISA methodology for detection of antibodies bound to immobilized TFPI. To date no anti-TFPI monoclonal antibodies have been detected with any of these three assays in spite of reasonable sensitivity demonstrated using rabbit antisera as positive controls.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH-07016-01-LHT

PERIOD COVERED

August 1991-August 1992

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

Use of Polyclonal Antisera to Tissue Factor Pathway Inhibitor.

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

Richard M. Lewis, PhD, Chemist,
Mary Catherine J. Schneider, MS, Biologist

COOPERATING UNITS (if any)

LAB/BRANCH

Hemostasis and Thrombosis Laboratory

SECTION

INSTITUTE AND LOCATION

CBER, Building 29

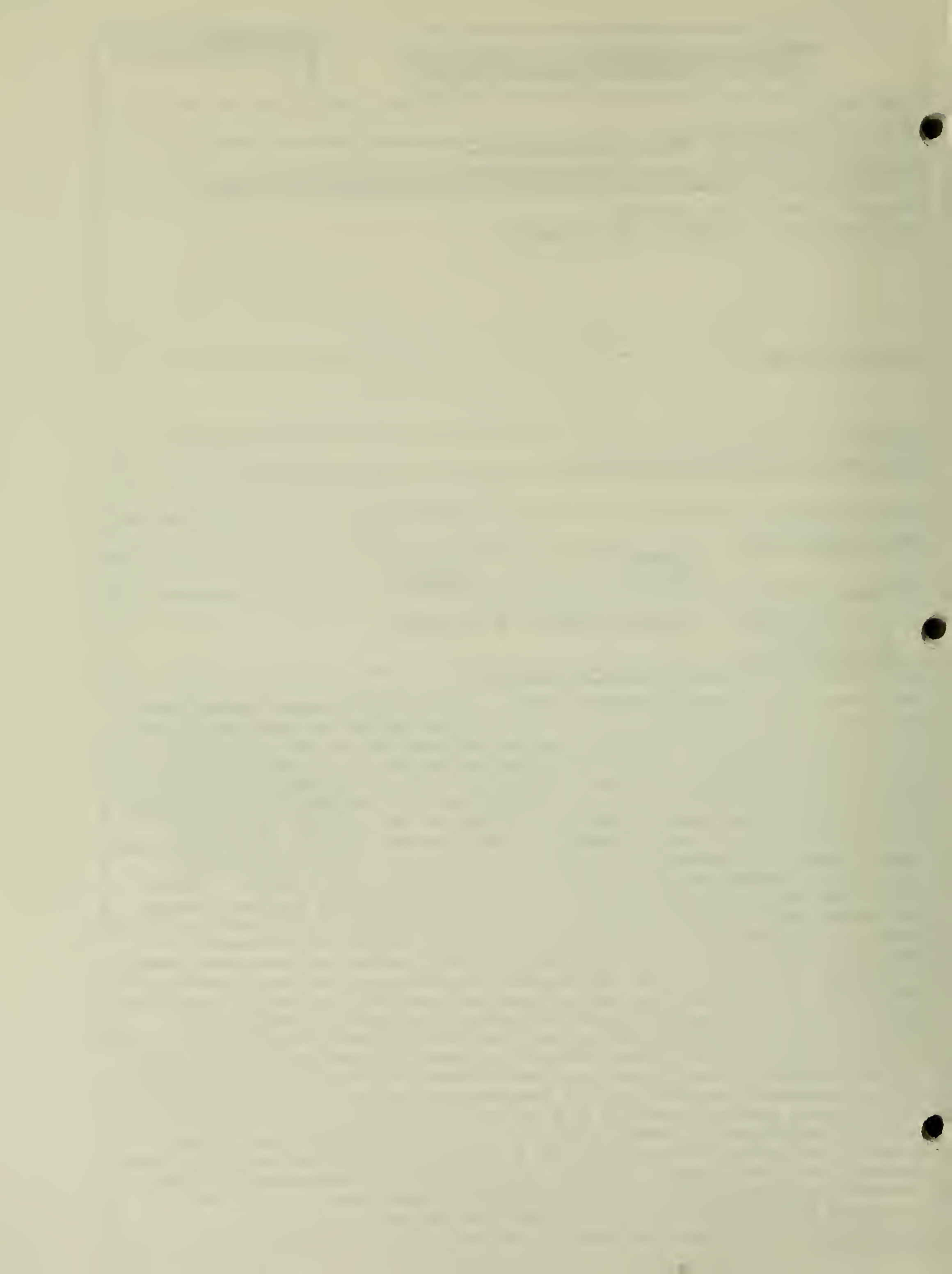
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

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

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

The tissue factor pathway inhibitor (TFPI) is a circulating plasma protein which can inhibit Factor Xa (F Xa) activity. The TFPI-Xa complex can then bind to the Factor VIIa-tissue factor (VIIa-TF) complex and block the activation of Factor X (F X) and Factor IX (F IX). VIIa-TF initiates the series of reactions referred to as the extrinsic coagulation cascade. Hemophilia A (Hem A) and Hemophilia B (Hem B) plasmas are deficient in Factor VIII (F VIII) and F IX, respectively. Even though these components are commonly thought to be part of the intrinsic coagulation cascade, the clotting time of hemophilic plasma becomes prolonged more rapidly than normal plasma with increasing dilution of tissue factor. In our experiments, rabbit brain thromboplastin (Tp) was used as a source of tissue factor. Because TFPI blocks both F Xa and the VIIa-TF activation of F X in the extrinsic pathway and because neutralizing antibodies to TFPI could increase the activation of F X in plasma, we hypothesized that neutralization of TFPI activity might correct the "dilute thromboplastin" prothrombin time (dil Tp) in normal and hemophilic plasma. To approach this question, the immunoglobulin fraction (Ig) of rabbit antibodies to TFPI (HEPG2-derived) were isolated and shown to block TFPI activity in normal human serum. They also reacted with crude TFPI material in ELISA and Western blot analyses. Dilute Tp times of Hem A, Hem B and normal plasmas were tested after the addition of anti-TFPI Ig, normal rabbit Ig or buffer. Samples were assayed with various dilutions of rabbit brain Tp. Hem A, Hem B and normal plasma dil Tp times were all reduced by anti-TFPI Ig in a dose-dependent manner. The neutralization of TFPI allowed a greater production of F Xa activity which resulted in reduction of clotting time not only in normal plasma but in Hem A and Hem B plasmas as well. Moreover, at high Ig concentrations, Hem A plasma dil Tp time was reduced to times equivalent to normal plasma. These results emphasize the importance of F X as central to the coagulation process and suggest the importance of F VIII and F IX for proper function of the extrinsic coagulation pathway.



Summary of Laboratory Programs
Plasma Derivatives Laboratory
Division of Hematology

October 1, 1991 through September 30, 1992

Laboratory Chief: Donald L. Tankersley, M.S.

Principal Investigators:

Bennett Kaufman, Ph.D., Immunology Section
Andrew Shrake, Ph.D., Physical Biochemistry Section
Mei-ying W. Yu, Ph.D., Hepatitis Section

Other Investigators:

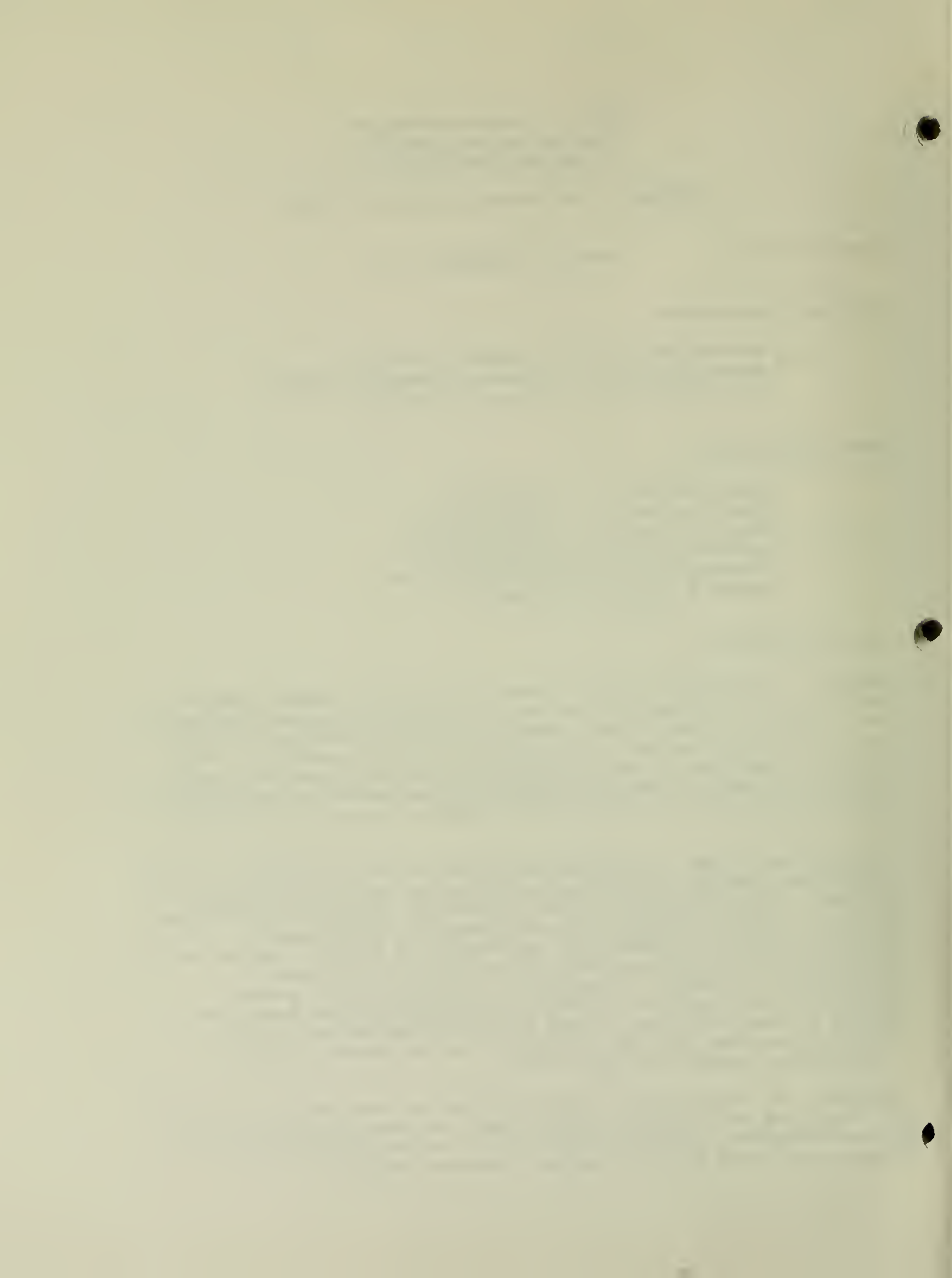
Douglas Frazier, B.S., Biologist
Zheng-ping Guo, M.D., Fogarty Fellow
Bobby L. Mason, M.S., Microbiologist
Lee Stevan, M.S., Microbiologist
Soonpin Yei, Ph.D., Senior Staff Fellow
Andrew M. Young, M.S., Chemist

Project Description:

RESEARCH. The Plasma Derivatives Laboratory maintains a strong research effort aimed at providing a better understanding of the products regulated by the laboratory in terms of their mechanism of action, possible deleterious effects, methods of analysis, and evaluation of product stability. This research is described in the Individual Intramural Project Reports appended. The research program of the Plasma Derivatives Laboratory was reviewed and evaluated by the Blood Products Advisory Committee during a site visit on June 23, 1992.

REGULATORY ACTIVITIES. The Plasma Derivatives Laboratory reviewed more than 200 different IND applications, many with numerous supplements, during the past year. During the past 9 months (October 1991 through June 1992), final action was taken on 36 product or establishment license amendments. Amendments included new methods for manufacture and analysis, new indications and formulations for existing products, and significant changes in manufacturing facilities. At the present time, there are 58 pending applications and amendments for which the Plasma Derivatives Branch has primary responsibility, most of them received within the last year. Laboratory personnel will have participated in the inspection of ten establishments during this fiscal year.

PERSONNEL. Dr. Soonpin Yei, a Senior Staff Fellow, left CBER in March, 1992. Dr. Zheng-ping Gao, a Fogarty Fellow, began a two-year appointment to the Plasma Derivatives Laboratory in March 1992. Dr. Basil Golding will be transferring to the Plasma Derivatives Laboratory shortly.



Publications, Abstracts and Oral Presentations

Kaufman BM. "Immortalization of cells in culture." Annual Meeting of the National Capitol Area Branch, Tissue Culture Association, Gaithersburg, MD, October 7, 1991 (abstract and oral presentation).

Shrake A, Ross PD. Origins and consequences of ligand-induced multiphasic thermal protein denaturation. Biopolymers, in press.

Shrake A, Ross PD. Origins of ligand-induced multiphasic protein denaturation. Abstracts of the 12th IUPAC Conference of Chemical Thermodynamics and the 47th Calorimetry Conference (Joint Meeting), Snowbird, Utah, August 16-21, 1992.

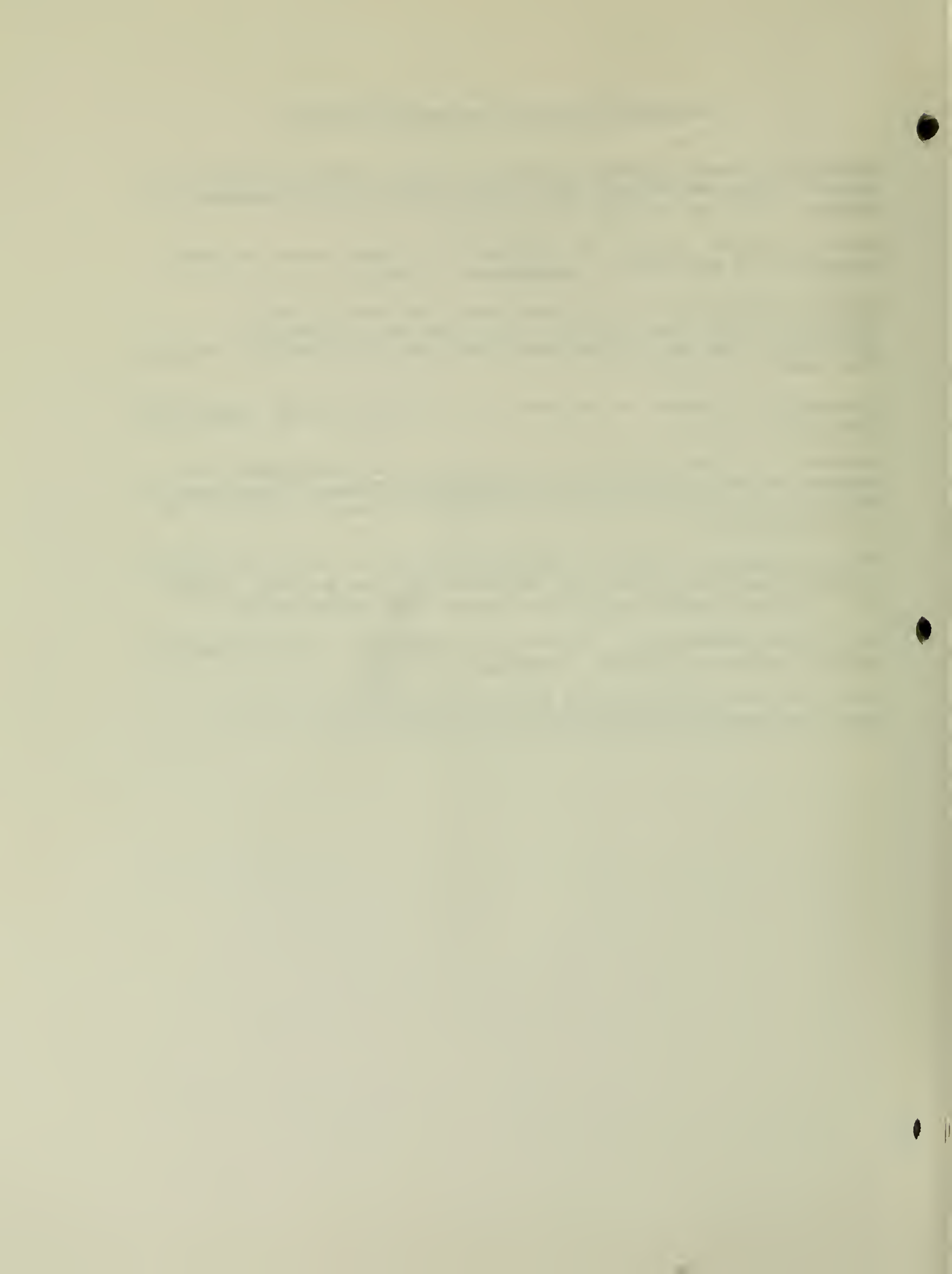
Tankersley DL, Finlayson JS. Housedust mite allergens and IgG. Lancet 339, 1364 (1992) (letter).

Tankersley DL. Dimer complex formation by the use of pooled human IgG. Symposium on the Role of Intravenous Gammaglobulin Therapy in Regulation of Immune Responses; Stockholm University, Stockholm, Sweden, October 21, 1991 (oral presentation).

Troy A, Kaufman BM, Clower M. Analysis of potatoes for residues of paraquat by enzyme immunoassay. Laboratory Information Bulletin No. 3641, February 1992. Office of Research Activities, Food and Drug Administration.

Yei S, Yu MW, Tankersley DL. Partitioning of hepatitis C virus during Cohn-Oncley Fractionation of Plasma. Transfusion, in press.

Yu MW. Structure and function of human albumin. Rivista Italiana di Nutrizione Parenterale ed Enterale 9:179-185 (1991).



Individual Intramural Project Report
October 1, 1991 through September 30, 1992

Project Title: Physical and Biochemical Characterization of Plasma Derivatives and Related Proteins and Materials

Principal Investigator: A. F. Shrake, Research Chemist, PDL, DH, CBER

Others: D. J. Frazier, Biologist, PDL, DH, CBER
A. M. Young, Chemist, PDL, DH, CBER
P. D. Ross, Research Chemist, LMB, NIDDKD, NIH

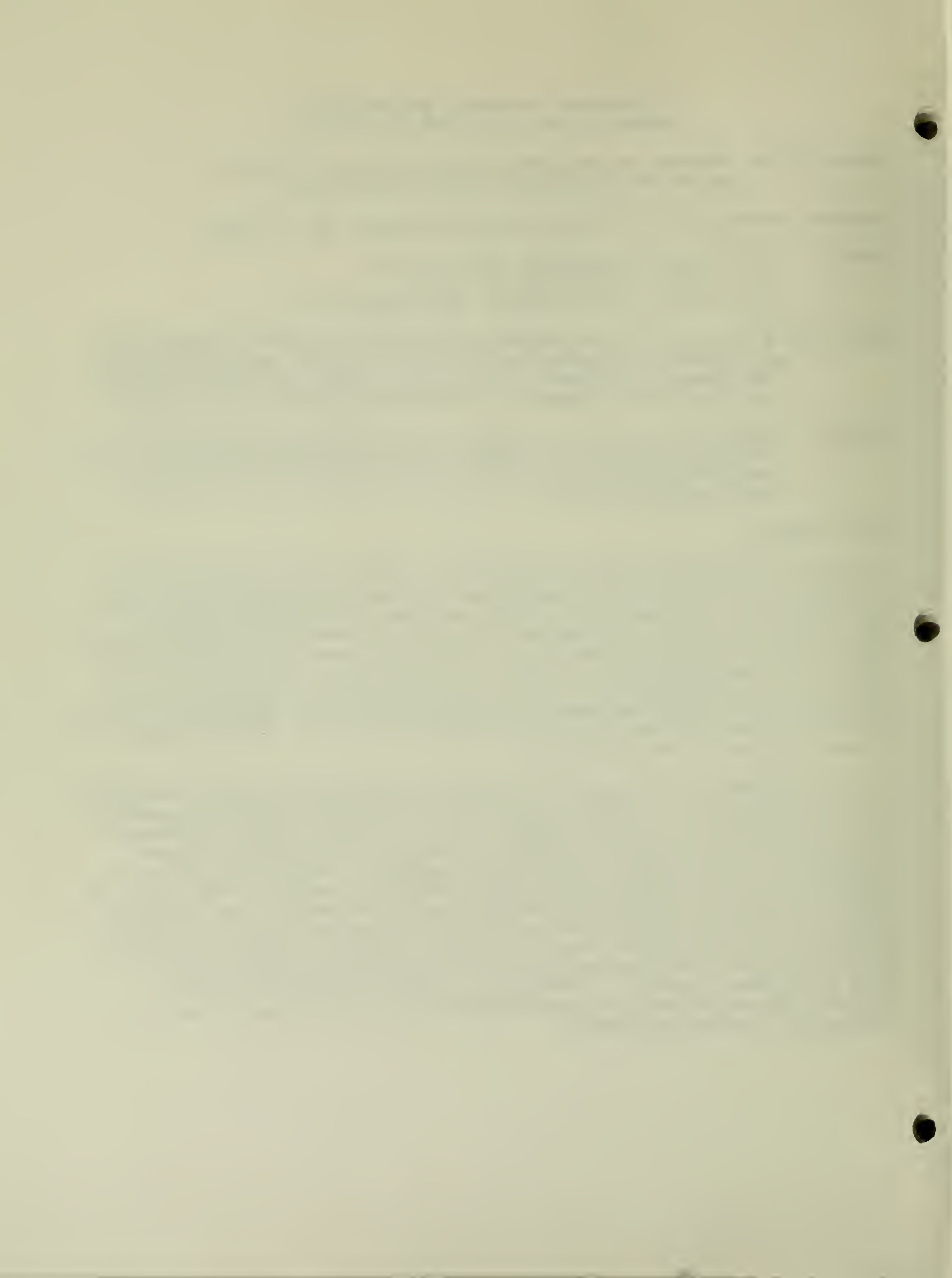
Objectives: A basic goal of this research is to provide an improved understanding of the products regulated by this laboratory with ramifications that are generally applicable to all protein systems and related materials in terms of both fundamental research and regulatory/product issues.

Methods: During the past year the studies involved computer modeling and utilized a differential scanning calorimeter, which was also calibrated and validated during this fiscal year, in addition to a photodiode array spectrophotometer.

Major Findings:

The previously developed thermodynamic model for ligand-induced biphasic protein denaturation, which was based on experimental observations and which was developed on the basis of the linkage between simple two-state protein denaturation and ligand binding equilibria, was extended to explain the number of denaturation peaks that occurs. Major ramifications of this are that (a) there is a possibility for ligand-induced multiphasic denaturation of a single cooperative unit and (b) the presence of only two maxima in a thermogram for a protein with multiple sites is not due to the exclusive denaturation of unliganded and fully liganded species with an absence of contributions from the denaturation of partially liganded species as proposed by others. (Shrake and Ross)

A study of the thermal denaturation of albumin by differential scanning calorimetry has been undertaken in an effort to probe the domain substructure of the protein, which is comprised of three major domains, and to elucidate interactions between the domains. We have found a profound pH dependence of the thermally induced denaturation of human albumin. We have also commenced a study of the effects of chloride binding on the temperature induced unfolding of bovine albumin (BA) in an effort to explain the effects of saturating levels of chloride on the thermograms. We have also prepared peptic fragments of bovine albumin with the free sulfhydryl blocked by L-cystine (cys-BA) that correspond to approximate complementary halves of the protein and that associate to form a complex with properties similar to those of the parent molecule. Furthermore, we have observed substantially different behavior between the thermal denaturation of BA and that of cys-BA at alkaline pH. (Shrake and Frazier)



Proposed Course:

The study of the thermal denaturation of domains within native albumin will continue. Particular attention will be paid to using changes in pH to perturb domain structure, and the effects of chloride binding will be explored. Emphasis will be placed on the denaturation of fragments, in particular those containing intact structural domains. Ultimately we would like to obtain information concerning the energetics of domain-domain interactions within albumin.

Our search for an appropriate membrane osmometer and suitable semipermeable membranes will continue in order to carry on the characterization of the colloidal plasma volume expanders, particularly in terms of number average molecular weight.

In order to demonstrate that ligand-induced biphasic denaturation is a general phenomenon and can also occur when the protein is unfolded with strong denaturants, we will continue to look for a small protein that denatures as a single cooperative unit in the absence of ligand that is suitable for such a study.

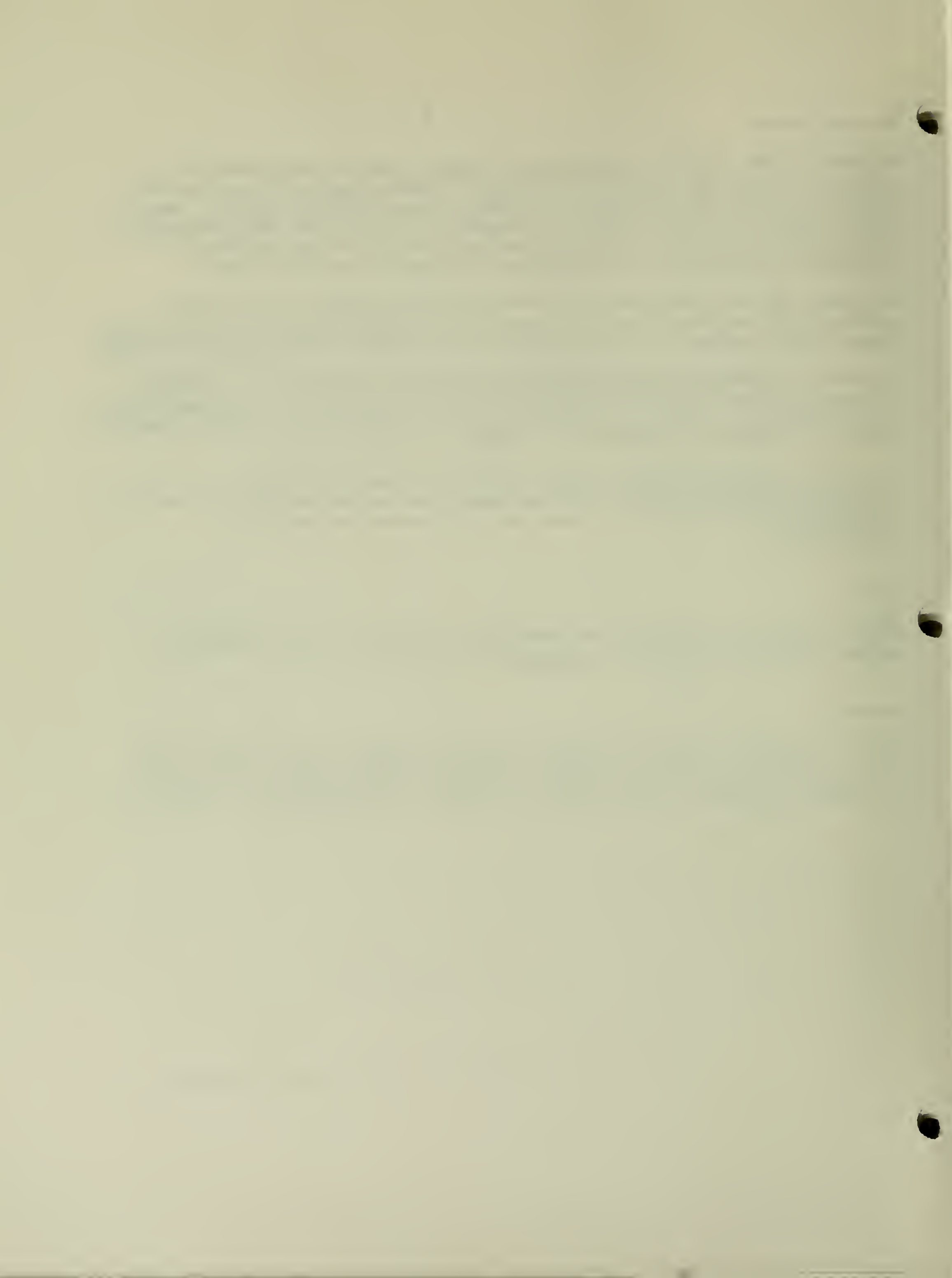
The search for a model protein system suitable for studying protein unfolding/refolding continues. With an appropriate system, equilibrium and kinetic techniques will be utilized to investigate the presence and nature of intermediates.

Paper:

Shrake, A, Ross, PD. Origins and Consequences of Ligand-Induced Multiphasic Thermal Protein Denaturation. Biopolymers, in press.

Abstract:

Shrake A, Ross PD. Origins of Ligand-Induced Multiphasic Protein Denaturation. Abstracts of the 12th IUPAC Conference of Chemical Thermodynamics and the 47th Calorimetry Conference (Joint Meeting), Snowbird, Utah, August 16 - 21, 1992.



Individual Intramural Project Report
October 1, 1991 through September 30, 1992

Project Title: Immunology of Viral Infections

Principal Investigator: Bennett Kaufman, Senior Staff Fellow, PDL, DH, CBER

Others: L. Stevan, Microbiologist, PDL, DH, CBER
K. Eckels, Chief, Biologics Research, WRAIR
P. Summers, Microbiologist, Biologics Research, WRAIR

Objectives: Gain understanding of the interaction of viruses with the immune system during infection so as to design effective vaccines that do not utilize the infectious agent.

Methods: A variety of procedures are employed, including chromatography (ion-exchange and affinity) for the separation and purification of antibodies and other proteins and peptides, cell culture for the production of hybridomas, solid-phase enzyme immunoassay (ELISA) for the screening of antibody-containing samples, and electrophoresis and western blotting for the analysis of proteins. Mice are used for ascites production and hybridoma development.

Major Findings:

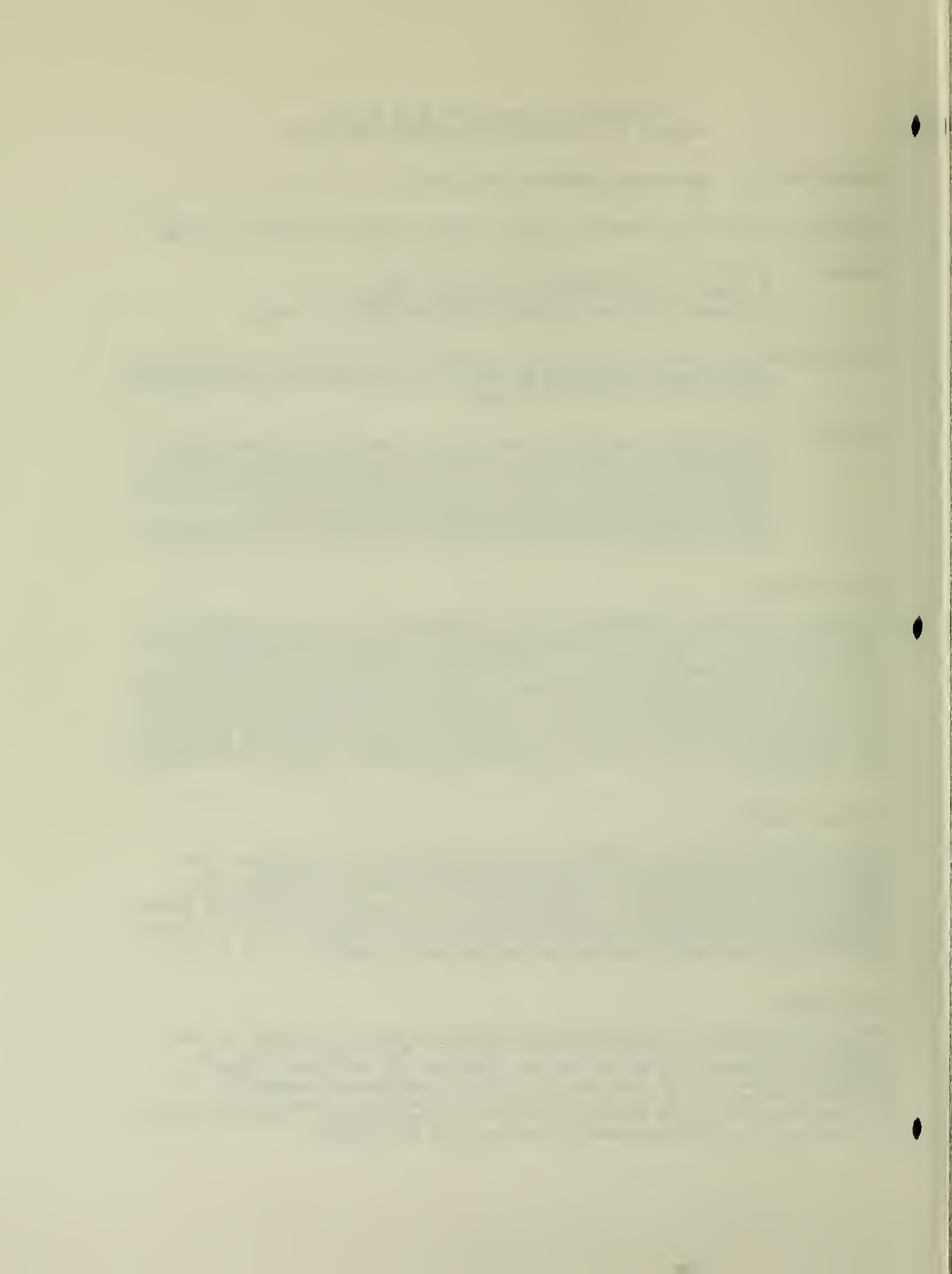
No stable hybridomas secreting antibody directed against the 4G2 immunogen have been obtained from the 250 hybrids produced in the 12 fusions performed during this year of the project. The 4G2 epitope of Japanese Encephalitis Virus (JEV) envelope glycoprotein is heat stable; however, its immunological reactivity with monoclonal antibody 4G2 is lost upon denaturation with either mercaptoethanol or dithiothreitol. E-glycoprotein does not need to be boiled before polyacrylamide electrophoresis, because its electrophoretic behavior and immunological reactivity in western blotting appear unaffected by heating. The E-glycoprotein can be partially digested by trypsin, with the retention of 4G2 reactivity.

Proposed Course:

Because the antiidiotype approach to the production of an antiflavivirus vaccine was not successful, an alternative approach was initiated. The cross-reactive and cross-protective epitope on flavivirus E-glycoprotein identified by the monoclonal antibody 4G2 will be identified utilizing enzyme digestion of E-glycoprotein, followed by sequencing of the reactive epitope. The peptide representing the protective epitope may be employed as a subunit vaccine effective against members of the flavivirus family.

Future Plans:

The tryptic fragment of E-glycoprotein containing the 4G2 epitope has been shown to be 17-18 kiloDaltons in size. However, the actual size of the epitope is unknown. The actual epitope size will be estimated either by further enzymatic and/or chemical digestion of the larger fragment to determine how small a piece will still retain immunologic reactivity, or by the determination of the reactivity of overlapping synthetic peptides spanning the region of the E-glycoprotein containing the 4G2 epitope.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 BH 05027-01-LPD

PERIOD COVERED
October 1, 1991 through September 30, 1992

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

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

B. Kaufman, Senior Staff Fellow, Plasma Derivatives Laboratory, DH, CBER
K. Eckels, Chief, Biologics Research, WRAIR
P. Summers, Biologics Research, WRAIR

COOPERATING UNITS (if any)
Dept. of Biologics Research, WRAIR

LAB/BRANCH
Plasma Derivatives Laboratory, DH

SECTION

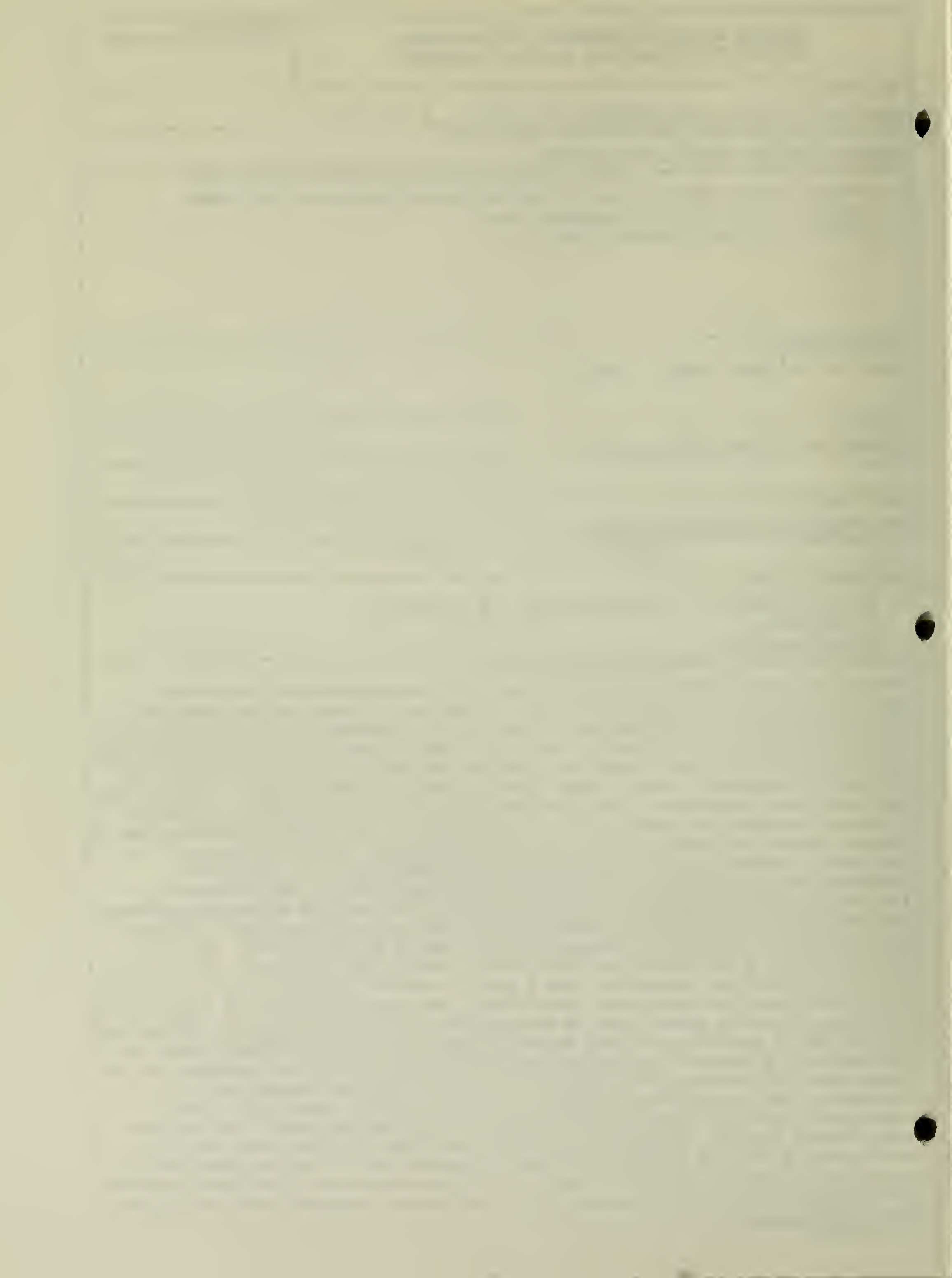
INSTITUTE AND LOCATION
DH, CBER, FDA, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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

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

The goal of this project is the production of a cross-protective flavivirus vaccine, based on the epitope identified by the murine monoclonal antibody 4G2. This epitope appears to be present on the 50-60 kD envelope (E) glycoprotein of all members of the Flavivirus family. Initially, the production of a murine monoclonal antiidiotype antibody was attempted. The 4G2 antibody, or its Fab fragment, was used as an immunogen in mice, either alone or coupled to BSA or KLH. Fusions were performed using splenocytes from immunized animals, and the resultant hybridomas screened for reactivity against 4G2. Testing of over 700 hybrids resulting from 30 fusions yielded no reactive clones. In a new approach, the 4G2 antibody will be employed to determine the exact cross-protective epitope on E. E will be fragmented by enzyme and/or chemical means, the reactivity of the fragment(s) followed by electrophoresis and western blotting, and reactive fragments sequenced. The 4G2 epitope as displayed on both Japanese Encephalitis Virus (JEV) and West Nile virus (WN) is stable to heating at 100 °C for up to six minutes. We have determined that a two-minute heat treatment at 100 °C will inactivate up to 8 logs of viral infectivity; therefore, such a step is used to inactivate the virus so it can be used under non-containment conditions. The dengue virus and WN epitopes are stable to denaturing agents such as mercaptoethanol and dithiothreitol, whereas the JEV epitope is sensitive to these denaturing agents. However, JEV was chosen as the source of E because large quantities of purified virus can be produced, and an animal model for testing the protective efficacy of vaccine candidates is available. The electrophoretic mobility and western blot reactivity of JEV E is unchanged by boiling before electrophoresis, so the heating step is not employed during these studies. Western blot analysis has shown that 4G2 reactivity is retained by a 17-18 kD tryptic/chymotryptic fragment of E. The E-glycoprotein is quite susceptible to trypsin cleavage, with epitope reactivity lost after exposure to 0.5 mg/ml trypsin for 4 hours at 37 °C; the optimum digestion time has not yet been determined.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 05014-06-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Origins of Ligand-Induced Multiphasic Thermal Protein Denaturation

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

PI: Andrew F. Shrake, Ph.D., Chemist, PDL, CBER, FDA

Other: Philip D. Ross, Ph.D., Chemist, LMB, NIDDKD, NIH

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health

LAB/BRANCH

Plasma Derivatives Laboratory

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.25		

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A thermodynamic model for ligand-induced biphasic thermal protein denaturation, which derives from experimental studies of the thermally induced denaturation of human albumin in the absence and presence of lower, intermediate, and higher affinity ligands [J. Biol. Chem. 263, 15392-15399 (1988)], was developed previously [J. Biol. Chem. 265, 5055-5059 (1990)]. Such biphasic denaturation does not relate to protein domain substructure since it can occur with a protein that undergoes simple two-state denaturation in the absence of ligand. If the affinity of the protein for a ligand is great enough and if initially the protein is subsaturated with ligand, biphasic denaturation can occur. We have developed a thermodynamic description of the linkage between the equilibria for simple two-state thermal denaturation and ligand binding in which the number of cusps present in the thermogram correlates with the number of resolved steps in the plot of saturation level of remaining native protein vs. temperature (i.e. the thermal binding curve). The form of this thermal binding curve is a function of the number of ligand binding sites on the protein, the magnitudes of the association constants, and the total ligand and total protein concentrations. As a result, the model indicates a possibility for multiphasic denaturation of a single cooperative unit. The presence of only two maxima in a thermogram for a protein with multiple sites on the native species derives from the form of the thermal binding curve, which in this case is a single step sigmoidal plot, and <u>not</u> from the predominant denaturation of unliganded and fully liganded native species. In addition, it is shown that, in general, the contributions from the denaturation of individual native protein species are decidedly non-two-state in character thereby indicating that simple deconvolution should not be carried out. In general, the interaction of ligand with denatured protein has little effect on the occurrence of biphasic denaturation although it may substantially modify peak shape and denatuaration temperature(s).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 05024-02-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Investigations of Protein Substructure

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

PI: Andrew F. Shrake, Ph.D., Chemist, PDL, CBER, FDA

Other: Douglas J. Frazier, B.S., Biologist, PDL, CBER, FDA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Plasma Derivatives Laboratory

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.65		

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

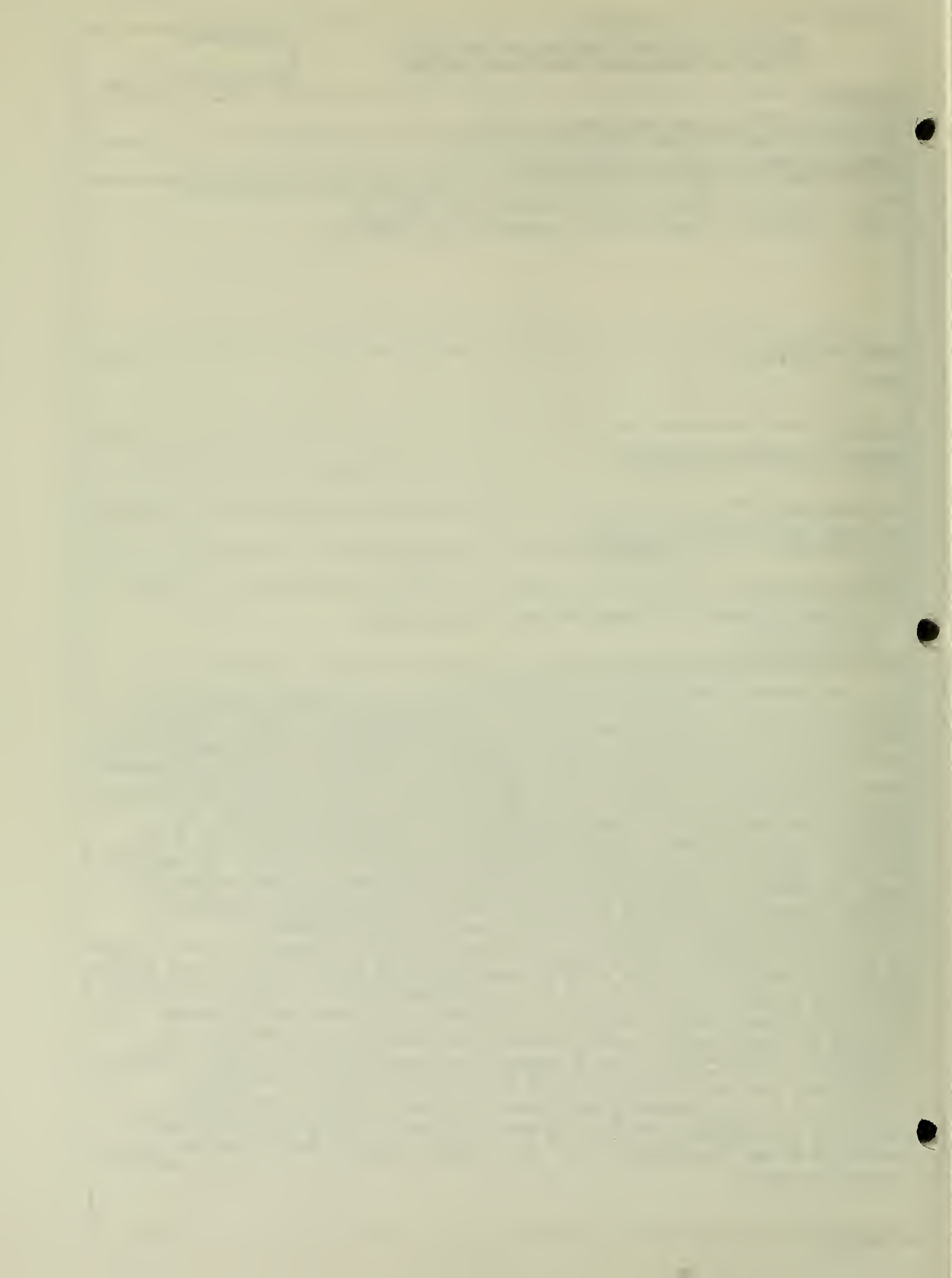
☐ (a2) Interviews

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

The thermal denaturation of bovine albumin (BA) was originally studied by Privalov at low ionic strength and at pH 7.0 exclusively [Mol. Biol. (Mosc.) 19, 1072-1078 (1985)]. Privalov was able to adequately deconvolute his thermograms for BA and a tryptic fragment into sums of two-state envelopes, which correspond to the unfolding of the constitutive domains; albumin is comprised of three major domains.

Initially we investigated the effect of pH at low ionic strength on the thermal denaturation of human albumin (HA) from pH 3.7-11.0 and generally found two major peaks although at pH's 4.0, 4.3, and 7.5, single peaks obtained. The denaturation temperatures of these peaks showed a marked pH dependence. This effect of pH on the thermal unfolding behavior is reversible since at pH 5.65 the two major denaturation peaks were unchanged by pH 5.65 → 3.7 → 5.65. In addition, over the range pH 5.5-10.0, there was a smaller endotherm at higher temperature, which was ascribed to a small amount of HA stabilized through the binding of a residual amount of long-chain fatty acid. At pH 5.5 the thermal denaturation process was reversible for the first endotherm but not the second.

Due to the relative facility in obtaining proteolytic fragments of BA, we opted to study the unfolding of this albumin. The effect of chloride binding was studied at pH 4.0 since the two major peaks are most separated here in comparison with the separation at pH's 3.5 and 5.3, the other pH's studied; in the range of 30-50 mM Cl^-, which is where significant Cl^- binding begins, the peaks begin to merge. From BA with the free sulfhydryl blocked by reaction with L-cystine (cys-BA), we prepared two complementary pepsin fragments, PA (307-581) and PB (1-306), which associate at alkaline pH to form a complex with some properties similar to those of native BA. The thermograms of PA+PB at pH 4.0 (not associated) and pH 8.6 (associated) both could be approximated as the sum of those of the isolated fragments at the corresponding pH's thereby suggesting little interaction between domains in the complex at pH 8.6. However, the most striking effect was the difference in denaturation between BA and cys-BA at pH 8.6; the thermogram for unmodified BA was comprised of three major peaks whereas that for cys-BA consisted of only two peaks.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 BH 05015-10-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Physical Characterization of Colloidal Plasma Volume Expanders

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

PI: Andrew F. Shrake, Ph.D., Chemist, PDL, CBER, FDA
Other: Andrew M. Young, M.S., Chemist, PDL, CBER, FDA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Plasma Derivatives Laboratory

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, Bethesda, MD 20892

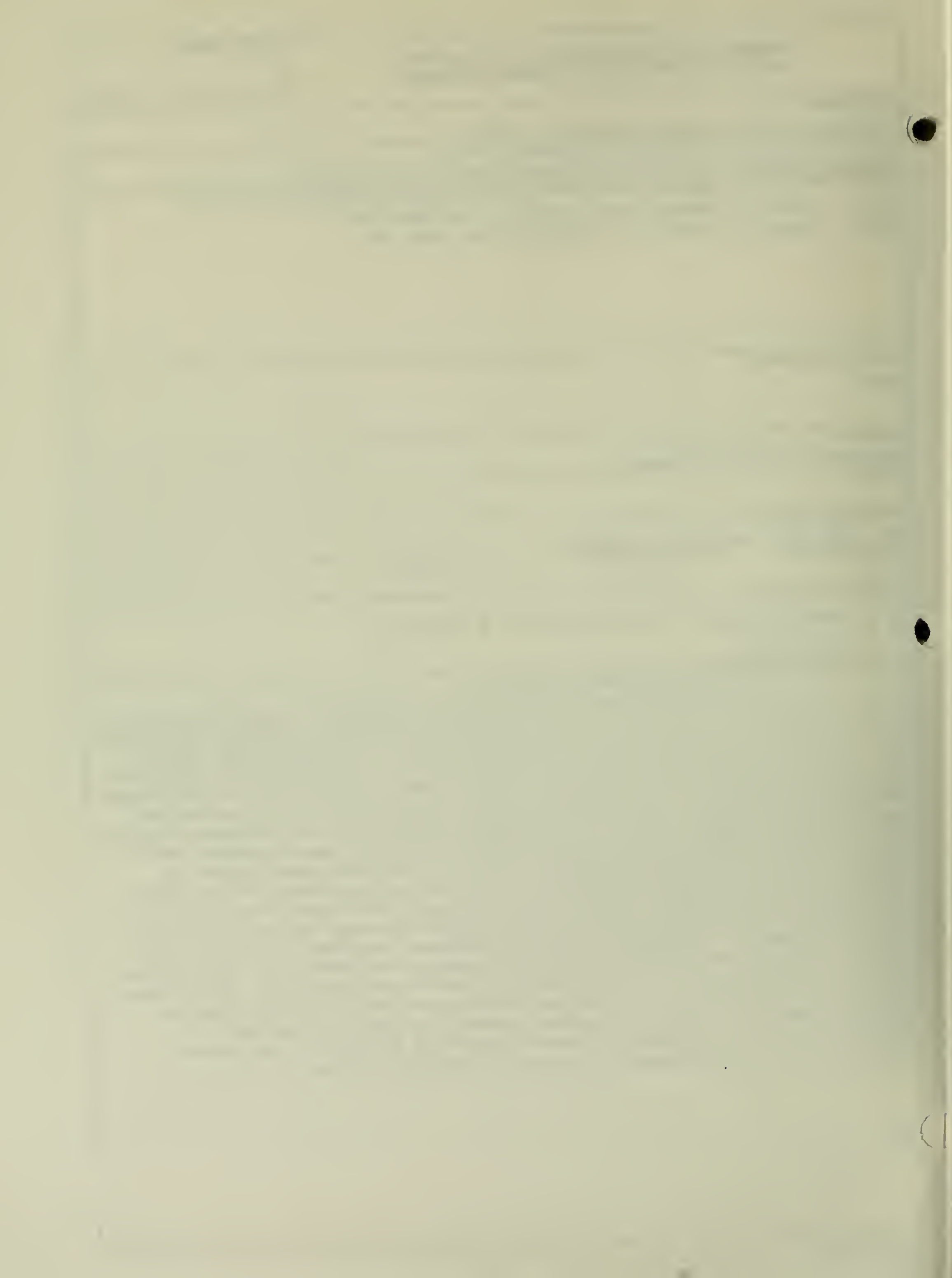
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.45		

CHECK APPROPRIATE BOX(ES)

☐ (a) 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 quest for an acceptable replacement for the defunct Knauer, Model 01.00 membrane osmometer continues. We have evaluated two Gonotec, Model 090B osmometers and found difficulty with both in obtaining reproducible readings when applying known hydrostatic pressures with the calibration rod. Compounding our difficulty in procuring suitable instrumentation is the current unavailability of appropriate semipermeable membranes. Nevertheless, we are continuing our search since the number average molecular weight (M_n) data that we have obtained for Dextran 70 and Dextran 40 have demonstrated that the HPLC-gel permeation chromatography (GPC) methodology in the proposed USP Monographs for the Dextrans gives correct M_n values. Furthermore, membrane osmometry has permited an estimate of the upper limit of M_n for Hetastarch, $<46,700>_s$; only an upper limit could be measured due to the presence of low concentrations of lower molecular weight species that were membrane permeable. This value is proving extremely important in resolving the conflict among M_n values being reported for innovator Hetastarch by manufacturers applying for approval of a generic Hetastarch and being reported by the innovator itself; all such reported M_n values derive from some sort of HPLC-GPC method. Clearly obtaining M_n values by membrane osmometry is not only important in understanding the physical/physiological properties of such colloid volume expanders but is also relevant in resolving regulatory issues.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 05016-03-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Ligand-Induced Biphasic Protein Unfolding by Strong Denaturants

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

PI: Andrew F. Shrake, Ph.D., Chemist, PDL, CBER, FDA

Others: Douglas J. Frazier, B.S., Biologist, PDL, CBER, FDA

Andrew M. Young, M.S., Chemist, PDL, CBER, FDA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Plasma Derivatives Laboratory

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05		

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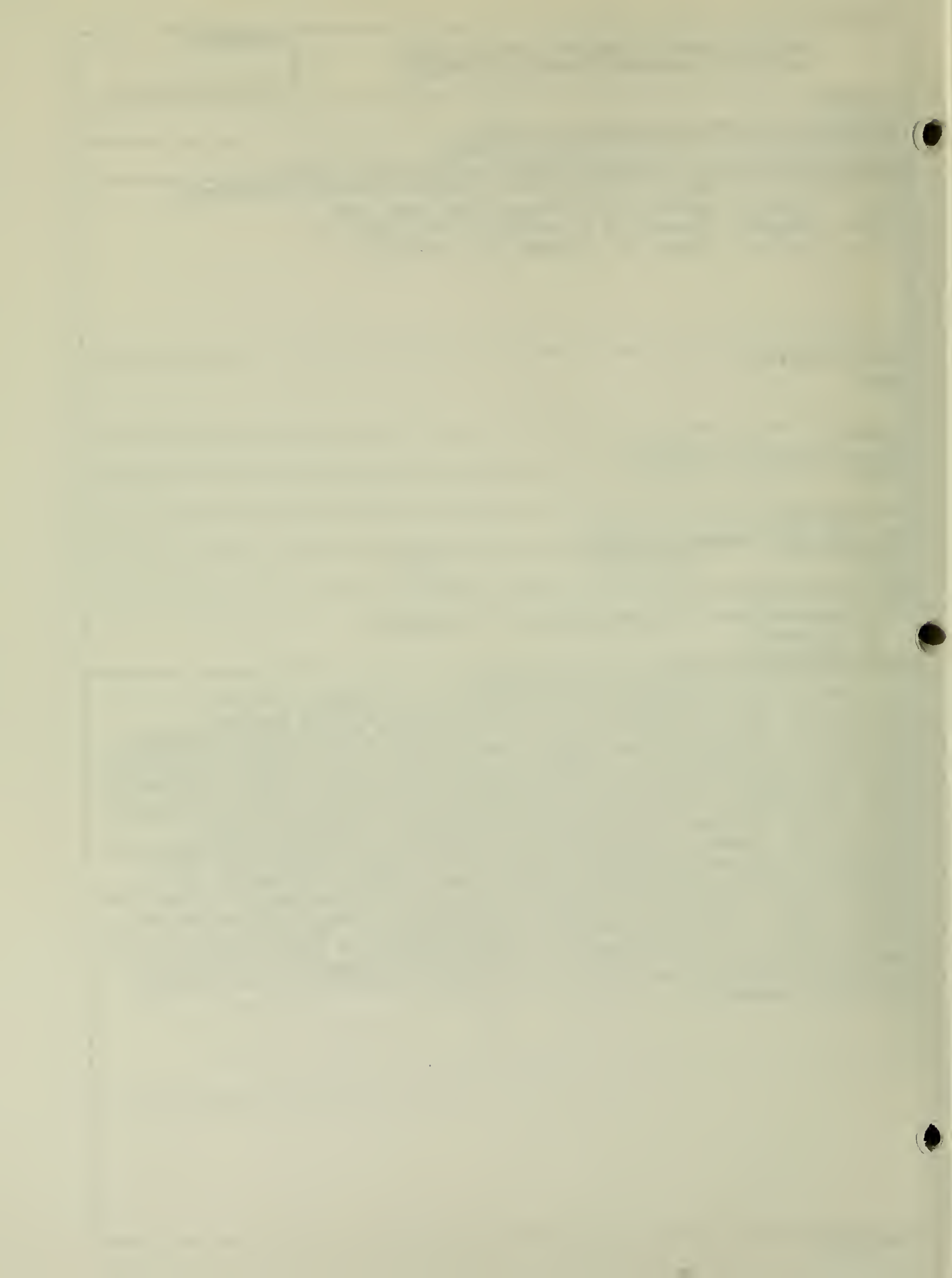
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

The ability of ligands to induce biphasic (or multiphasic) protein denaturation should be independent of the means used to effect protein denaturation. Thus, we began a study of the effect of ligands on the guanidine HCl-induced unfolding of human albumin. We anticipated that biphasic denaturation should occur in the presence of subsaturating levels of a high affinity ligand. A preliminary experiment with undefatted human albumin, which contained ~1.4 mol of bound high-affinity, endogenous, long-chain fatty acid/mol of albumin monomer and which corresponds to ~15% saturation, showed biphasic denaturation when monitored by UV protein difference spectroscopy and by changes in intrinsic protein fluorescence. However, on defatting human albumin and subjecting this protein to denaturation with guanidine HCl in the absence of ligand, we found that this ligand-free protein also undergoes biphasic denaturation; this behavior had been demonstrated earlier for bovine albumin by Khan et al. using urea [J. Biochem. 102, 313-317 (1987)]. Clearly this biphasic denaturation is <u>not</u> ligand-induced but rather relates to the domain substructure of the protein. We propose to look for a smaller protein that in the absence of ligand unfolds as a single cooperative unit when denaturing with strong denaturant but that has a ligand for which it has sufficient affinity to show ligand-induced biphasic denaturation when unfolding with strong denaturant.



Individual Intramural Project Report
October 1, 1991 through September 30, 1992

Project Title: Studies Related to Hepatitis B and C

Principal Investigator: Mei-ying W. Yu, Research Chemist, PDL, DH,CBER

Others: Soonpin Yei, Senior Staff Fellow, PDL, DH, CBER
Zheng-ping Guo, Forgarty Fellow, PDL, DH, CBER
Bobby L. Mason, Microbiologist, PDL, DH, CBER
Donald L. Tankersley, Chief, PDL, DH, CBER
J. S. Finlayson, Acting Director, DH, CBER
James W.-K. Shih, Microbiologist, CC, NIH
A. R. Neurath, The Lindsley F. Kimball Research Institute of NYBC
Wei-Mei Ching, Naval Medical Research Institute

Objectives: To understand the partitioning of hepatitis C virus during Cohn-Oncley fractionation of plasma, to detect hepatitis virus C RNA in plasma derivatives, to increase our understanding of the mechanism of infection by hepatitis B virus, and the mechanism by which infection may be prevented by immunoglobulin.

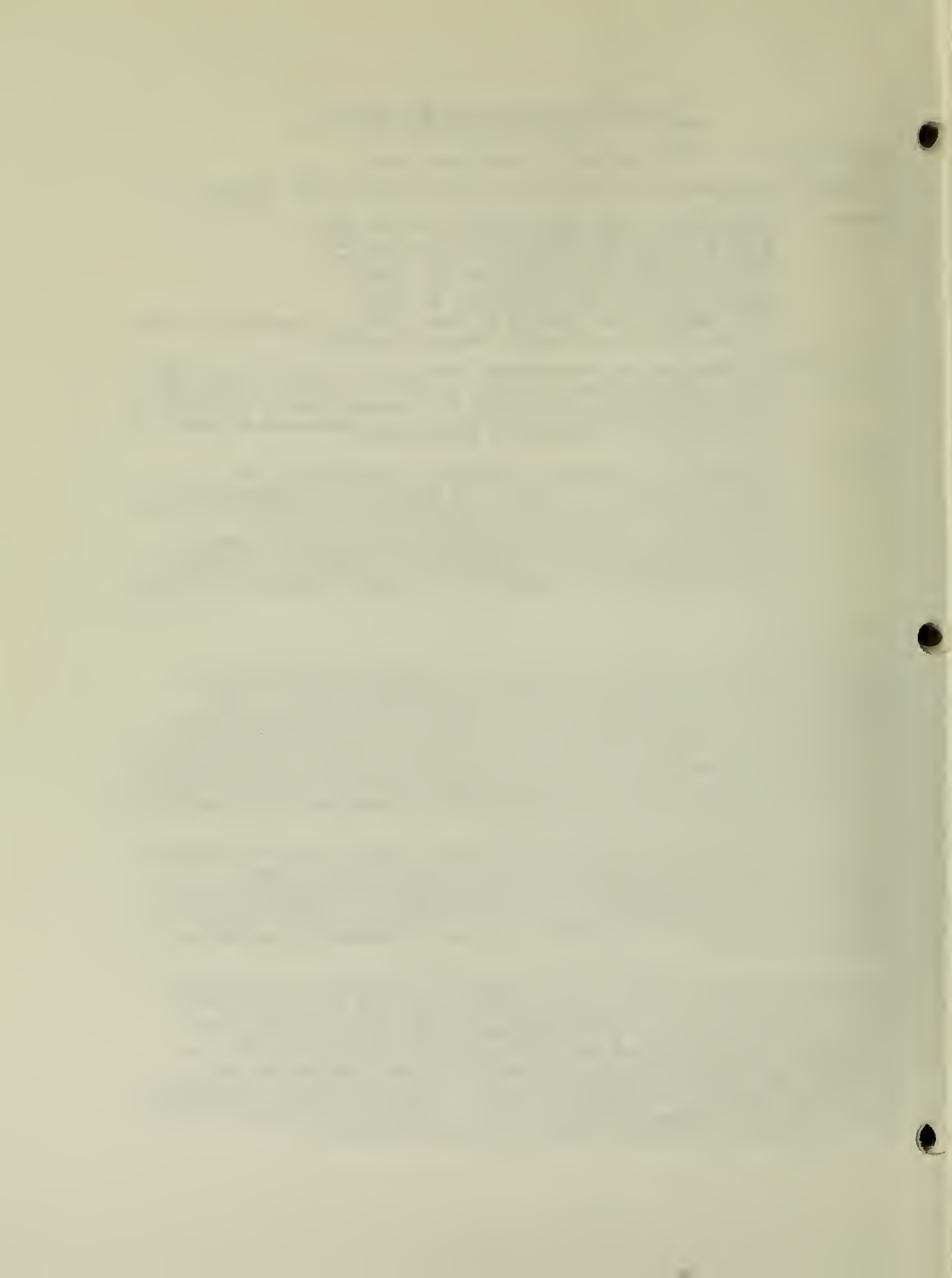
Methods: A variety of procedures, including ultracentrifuge, RNA extraction, reverse transcriptase reaction and polymerase chain reaction in a thermal cycler, and agarose gel electrophoresis are utilized in the detection of HCV RNA in plasma and its derivatives. Recombinant DNA techniques are used to prepare point mutated recombinant plasmids. The expressed proteins are purified by general protein purification techniques in quantity for binding studies. Chimpanzees are used in infectivity studies.

Major findings:

Our quantitation of HCV RNA by "nested" PCR method, which utilizes four primers derived from the highly conserved 5'-noncoding region of the HCV sequence, had a sensitivity comparable to the chimpanzee model. A pool prepared from 186 anti-HCV (anti-c100-3) reactive donations was assayed to contain 1.4×10^5 PCR units of HCV RNA/ml whereas a pool prepared from 2887 anti-HCV negative donations contained 1.6×10^3 PCR units/ml. By calculation, the unscreened plasma pool comprised of all the above donation would contain 1.0×10^4 PCR units/ml. Thus, anti-HCV screening decreased the viral load of the plasma pool by a factor of 6 (83%), which was comparable to the reported sensitivity of the anti-HCV screening test.

During Cohn-Oncley fractionation of the plasma pool derived solely from anti-HCV reactive donations, HCV RNA was found to partition mostly into cryoprecipitate, fraction I and fraction III, but a trace amount of HCV RNA was also detected in fraction II. A 3.4% solution of IgG prepared from this fraction II contained 30 PCR units/ml. Thus, the fractionation process leading to immune globulin resulted in an overall reduction in HCV RNA by a factor of 4.7×10^4.

HCV RNA was detected in one of 7 IGIV lots (from 7 different manufacturers) prepared from anti-HCV negative Source Plasma pool, and in 4 of 32 routine IGIV lots (from the same 7 manufacturers) prepared from unscreened plasma pools. Interestingly, 4 of 5 lots produced by one manufacturer from unscreened Source Plasma were positive for HCV RNA, whereas none of 4 lots produced from unscreened recovered plasma by the same manufacturer was positive. HCV RNA was also detected in all (2 lots each) of the intramuscular IG lots and older AHF lots assayed thus far. Although detection of HCV RNA does not necessarily equate with infectivity, substantial amounts of HCV RNA could be present in therapeutic doses of plasma derivatives.



The wild type recombinant preS1 peptide (rpreS1) was negative in its competition capability in Dr. Neurath's unique binding system of HBsAg to HepG2 cells. A mutant recombinant plasmid which can express a fusion protein containing a 90 amino acid mutant preS1 peptide in _E. coli_ has been successfully obtained. Upon Factor Xa digestion, this mutant rpreS1 peptide, which contains tyr^{12} tyr^{13} rather than a wild type phe^{12} phe^{13} and a C-terminal gly^{90}, was released. This mutant rpreS1 has been purified to homogeneity and its 18 N-terminal amino acid residues confirmed by amino acid sequence analysis. It can be ^{125}I-labeled directly because of the presence of the tyrosine residues.

The monoclonal anti-_d_ did not neutralize the HBV infectivity of the a_y_w subtype in a control chimpanzee. Thus, this monoclonal anti-_d_ is specific to a_d_ but not to a_y_ subtype and the epitopes recognized by this anti-_d_ may be a potential HBV vaccine.

Future plans:

Our preliminary results from the study of digestibility with RNase indicated that HCV RNA may be present as "intact" virus in plasma and as "naked" RNA in immune globulin products. If this can be further substantiated, the presence of HCV RNA in immune globulin products would be somewhat less alarming, since it would presumably not be infectious due to instability of RNA in immune globulin and the lack of replicating machinery for the virus. This may also explain the apparent safety of the immunoglobulin products. The ultimate test of infectivity (or lack thereof) will be carried out by infusing chimpanzees with immune globulin produced from the anti-HCV reactive plasma pool.

Utilization of primers derived not only from the 5' noncoding region but also from other portions of the HCV genome will be used in PCR to monitor the presence of HCV RNA in plasma derivatives. Furthermore, the possible disruption of the viral envelope in plasma by ethanol at various concentrations or by other virucidal or chromatographic procedures will be investigated.

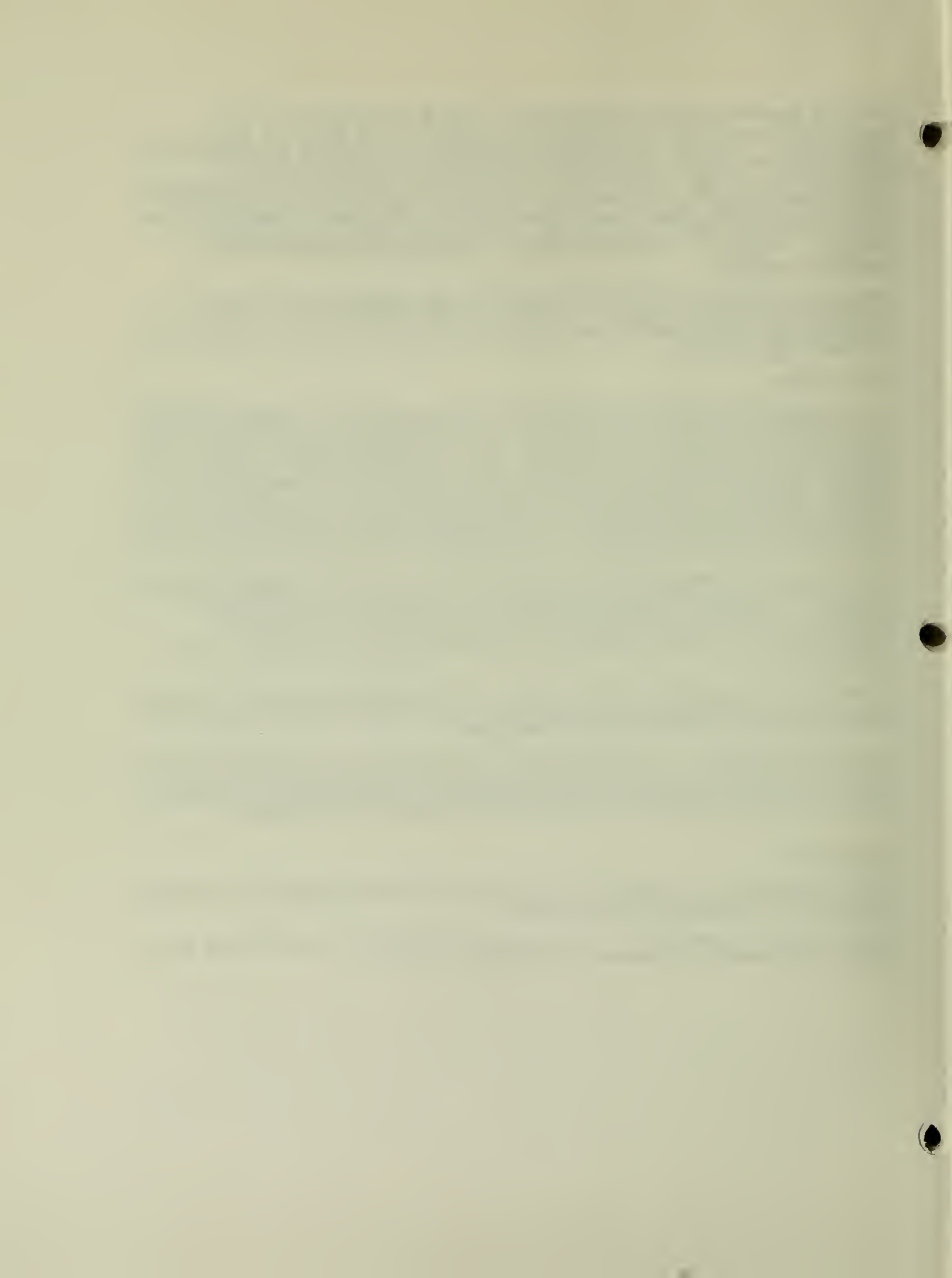
Detection and quantitation of HCV RNA in plasma derivatives will be continued. Those 186 anti-HCV reactive plasma samples will be individually titrated for HCV RNA titers by limiting dilution analysis.

The binding studies of a mutant rpreS1 to human liver plasma membranes will be continued in attempts to elucidate the complex nature of the interaction of virus with membrane receptors. The distribution of HBV receptors in various cell cultures and tissues of different species may also be examined.

Publications:

Yu MW. Structure and function of human albumin. Rivista Italiana di Nutrizione Parenterale ed Enterale 9: 179-85 (1991).

Yei S, Yu MW, Tankersley DL. Partitioning of hepatitis C virus during Cohn-Oncley Fractionation of Plasma. Transfusion, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 05028-01-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Partitioning of Hepatitis C Virus during Cohn-Oncley Fractionation of Plasma

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

Mei-ying W. Yu, Research Chemist, Plasma Derivatives Laboratory, DH, CBER
Soonpin Yei, Staff Fellow, Plasma Derivatives Laboratory, DH, CBER
Donald L. Tankersley, Chief, Plasma Derivatives Laboratory, DH, CBER

COOPERATING UNITS (if any)

LAB/BRANCH

Plasma Derivatives Laboratory, DH

SECTION

INSTITUTE AND LOCATION

DH, CBER, FDA, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

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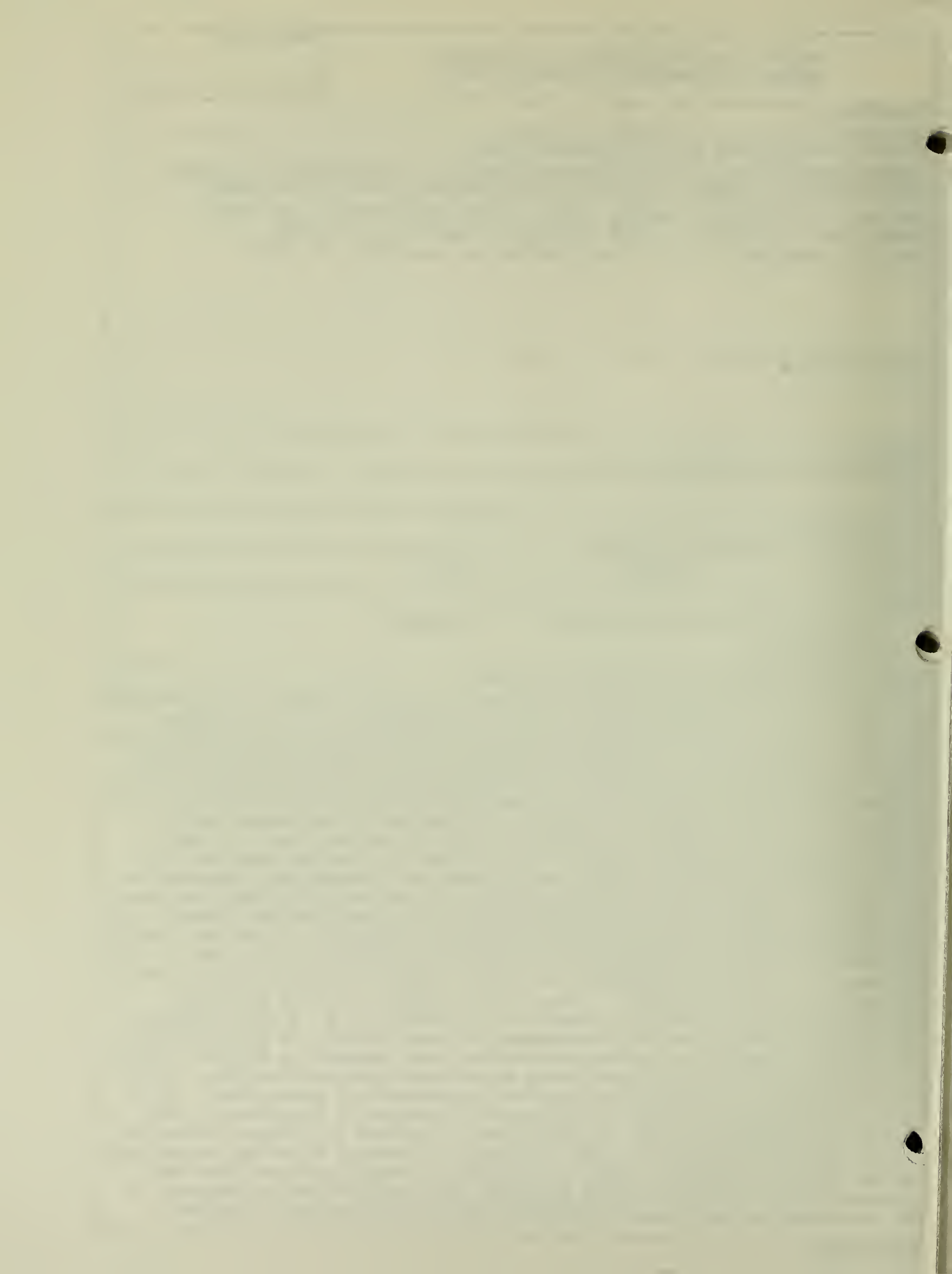
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Because of concern about the safety of immunoglobulins with respect to transmission of hepatitis C, we examined the partitioning of hepatitis C virus (HCV) during alcohol fractionation of a plasma pool prepared exclusively from anti-HCV reactive donations. Quantitation of HCV RNA was accomplished by nested polymerase chain reaction (PCR) at limiting dilution. One PCR unit was arbitrarily defined as the minimum amount of HCV RNA from which an amplified product could be detected. The sensitivity of the PCR assay for HCV RNA was determined by performing limiting dilution analysis on a sample of infectious plasma (H strain) known to contain $10^{6.5}$ chimpanzee infectious doses/ml. In our assay, this sample contained 1.4×10^6 PCR units of HCV RNA/ml. Thus, the PCR assay has a sensitivity comparable to the chimpanzee model. When 3,073 plasma donations from otherwise acceptable donors were tested for anti-HCV, 186 were repeatedly reactive and 2,887 were negative. A pool prepared from the anti-HCV reactive donations contained 1.4×10^5 PCR units of HCV RNA/ml, whereas a pool prepared from the negative dontaions contained 1.6×10^3 PCR units/ml. It can be calculated that a pool comprised of all 3,073 units would contain 1.0×10^4 PCR units/ml. Thus, in this instance, anti-HCV screening decreased the viral load of the plasma pool by a factor of 6. A 100-ml sample of the anti-HCV reactive pool was fractionated to immune globulin by the Cohn-Oncley procedure, and samples of the various fractions were analyzed for HCV RNA. Most of the HCV RNA was found in cryoprecipitate and in Cohn fractions I and III, but it was also detected in fraction II, used for immunoglobulin G preparations. A 3.4% solution of IgG prepared from this fraction II contained 30 PCR units/ml. The fractionation process leading to immune globulin resulted in an overall reduction in HCV RNA by a factor of 4.7×10^4. Although the presence of HCV RNA in the final product does not necessarily imply the presence of infectious virus, this work suggests that the safety of immune globulins with respect to HCV transmission is not due solely to partitioning of HCV away from the immunoglobulin fraction.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BH 05022-03-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

The Protectivity of an Anti-Subtype d Antibody against HBV Infection

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

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Bobby L. Mason, Microbiologist, Plasma Derivatives Laboratory, DH, CBER
J. S. Finlayson, Acting Division Director, DH, CBER
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2		

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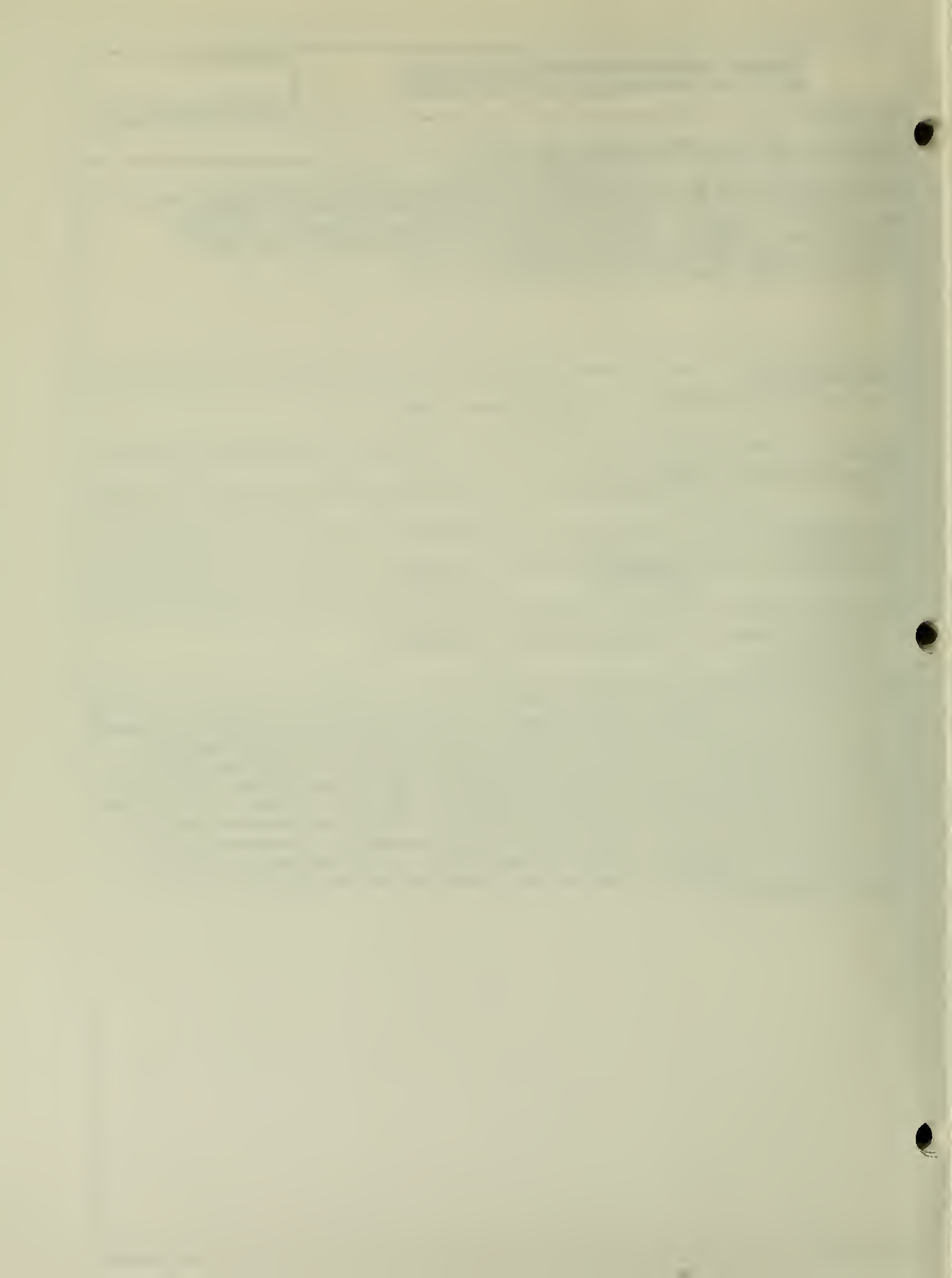
☐ (a1) Minors
☐ (a2) Interviews

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

As described in the previous annual report, a monoclonal anti-d antibody is capable of neutralizing the infectivity of hepatitis B virus (HBV) of the adw subtype in a susceptible chimpanzee. We have since inoculated a control chimpanzee with a mixture of this monoclonal anti-d antibody and HBV of the ayw subtype (1000 CID_{50}). The levels of HBV serological markers (HBsAg, anti-HBs and anti-HBc) and ALT liver enzyme were monitored with weekly blood specimens. HBsAg was detected beginning four weeks after inoculation, and the ALT level exceeded 2.5 times baseline at 13 weeks, peaking at 15 weeks. Thus, this monoclonal anti-d antibody is specific to ad but not to ay subtype and the epitopes recognized by this anti-d may be a potential HBV vaccine.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-BH-05021-04-LPD

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Expression and Characterization of Wild Type and Mutant PreS1 Peptides of HBsAg

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

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Zheng-ping Guo, Forgarty Fellow, Plasma Derivatives Laboratory, DH, CBER
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COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) Minors
☐ (a2) Interviews

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

As described in the previous annual report, the more recent preparations of our (wild type) recombinant preS1 peptide (rpreS1) contained two peptides which are only separable by SDS-PAGE. When this rpreS1 preparation was used as a labeled ligand by conjugating with ^{125}I-Bolton-Hunter reagent, no specific binding to either plasma membranes prepared from human hepatocytes or Hep G2 cells was observed. A sample of this rpreS1 preparation was sent to Dr. Neurath but tested negative in its competition capability in his unique binding system of HBsAg to HepG2 cells. Hence, point mutations were performed and produced a mutant recombinant plasmid which can express a fusion protein containing a 90 amino acid mutant preS1 peptide in E. coli. Upon Factor Xa digestion, this mutant rpreS1 peptide, which contains tyr^{12} tyr^{13} rather than a wild type phe^{12} phe^{13} and a C-terminal gly^{90}, was released. The mutant rpreS1 has been purified by mono-Q column chromatography, and 18 N-terminal amino acid residues have been confirmed by amino acid sequence analysis. This mutant rpreS1 can be labeled with carrier-free Na ^{125}I by means of the Iodogen method because of the availability of tyrosine residues. Binding studies are in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 BH-05029-01-LPD

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Detection of Hepatitis C Virus RNA by PCR in Plasma Derivatives

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

Mei-ying W. Yu, Reserch Chemist, Plasma Derivatives Laboratory, DH, CBER
Bobby L. Mason, Microbiologist, Plasma Derivatives Laboratory, DH, CBER
Donald L. Tankersley, Chief, Plasma Derivatives Laboratory, DH, CBER

COOPERATING UNITS (if any)

LAB/BRANCH
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

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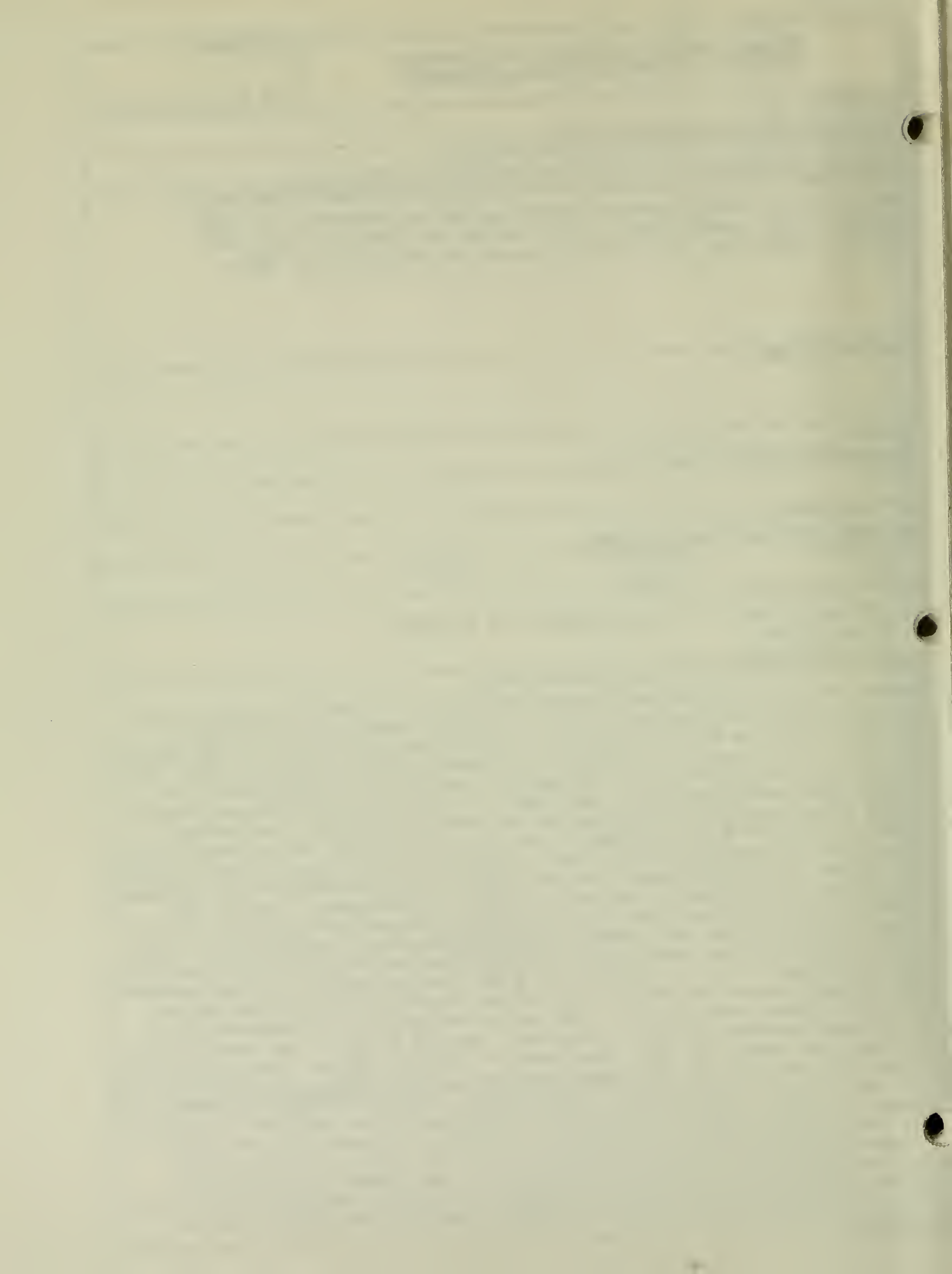
☐ (a1) Minors
☐ (a2) Interviews

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

Because the sensitivity of our "nested" PCR method is at least equivalent to the chimpanzee model, we proceeded to use the method to assay HCV RNA in plasma derivatives such as IGIV, IG, and AHF. An ultracentrifugal method (50,000 rpm for 3 h with a 70 Ti rotor, ca. 180,000 x g) was developed to concentrate "virus" in the pellet while at least 90% of the protein remained in the supernatant. We assayed seven experimental lots, produced by 7 manufacturers from Source Plasma (2,887 units) that had been screened for anti-HCV (anti-c100-3). HCV RNA was detected in one of these experimental lots, even though there was no evidence of hepatitis C transmission when this lot, along with the other six PCR-negative lots, was infused into 3 chimpanzees. We also analyzed 32 routine lots of IGIV (prepared from plasma not screened for anti-HCV) from the 7 manufacturers. HCV RNA was detected in four of these lots, all from one manufacturer (the same manufacturer whose experimental IGIV lot, produced from screened plasma, was positive). These five positive lots were determined by limiting dilution analysis to have from 10 to 250 PCR U/g IgG. It is of interest that 4 IGIV lots produced by this manufacturer from <u>recovered</u> plasma was negative for HCV RNA, whereas 4 out of 5 lots derived from Source Plasma were positive. One lot of VZIG prepared from recovered plasma by the same manufacturer was also negative. This may be due to the lower anti-HCV reactive rate, hence, the lower viral load, in recovered plasma. Two intramuscular IG lots and two older AHF lots were assayed and found to contain 30 and 260 PCR U/g IgG, and 0.1 and 4 PCR U/IU FVIII, respectively. One lot of Coagulation Factor IX (Human) (purified, heated in heptane) was assayed and tested negative. Preliminary data obtained from studies utilizing RNase A digestion suggest that the HCV RNA presented in immune globulins may be as a "naked" RNA rather than intact virus; this may explain how viral RNA can be present in some immune globulin products without transmitting hepatitis C to recipients. Detection and quantitation of HCV RNA and its digestibility by RNase A in plasma fractionation products will be continued.



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