Medical Center North Chicago IL 60064

Veterans Administration

In Reply Refer To: 556/11B

United States Nuclear Regulatory Commission
Material Licensing Section
799 Roosevelt Road
Glen Ellyn, IL 60137

Gentlemen:

Please amend our by-product material license number 12-10057-04, to include Kalkunte Srivenugopal, Ph.D., as an authorized user of licensed material for laboratory research, including animal studies.

Enclosed is his training and experience in handling radionuclides, the type of use, where the experience was gained, and the duration of each experience.

Dr. Srivenugopal has submitted a research proposal which was approved by our Radiation Safety Committee.

Sincerely yours,

Leonard C. Rogers
Medical Center Director

Enclosures:
Research Proposal
Curriculum Vitae
Approval of the Radiation Safety Committee

JAMES W. FLETCHER, M.D.
Director, Nuclear Medicine Service (115)
Veterans Administration
Washington, DC 20420





CONTROL NO. 87985

Proposal Received _____

INTRAMURAL GRANT APPLICATION

UNIVERSITY OF HEALTH SCIENCES/THE CHICAGO MEDICAL SCHOOL

Principal Investigator Kalkunte S. Srivenugopal, Ph.D. New Proposal X
Department Surgery Continuation NO
Project Title Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression in Septic Rat Liver

Total Requested (include itemized budget in your application) $ 5,000.00

| This Project Will Involve the Use of: (Check Yes or No) | YES | NO | Approved for Compliance (When Applicable |
|---|---|---|---|
| Human Subjects | ___ | X | _____ Chairperson, Institutional Review Board for the Protection of Human Subjects |
| Animals | X | ___ | _____ Chairperson, Animal Care Committee |
| Radioactive Materials | X | ___ | _____ Chairperson, Radioisotope Committee |
| Bio-Hazards | ___ | X | _____ Chairperson, Bio-Hazards Committee |

SIGNATURES:

K S Srivenugopal — 11.3.1988.
Kalkunte S. Srivenugopal, Ph.D. — Date
Principal Investigator

William Schumer M.D. — 11/3/88
William Schumer, M.D. — Date
Department Chairman

**********(Space Below for Administrative Use Only)**********

_____ _____
Grants Manager — Date

Grant Assignment No: _____

Project Funded: Yes ___
No ___

_____
BRSG, Program Director



CONTROL NO. 87385

Project Title: Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression in Septic Rat Liver

Principal Investigator: Kalkunte S. Srivenugopal, Ph.D.

BUDGET

| | | |
|---|---|---|
| A. | Equipment | |
| | Dot Blot Apparatus | $ 300.00 |
| | Ultraviolet Transilluminator | 900.00 |
| | Polaroid MP-4 Land Camera | 1,800.00 |
| B. | Supplies | |
| | Biochemicals. Radioisotopes and Restriction Enzymes | 1,500.00 |
| C. | Animals | |
| | Fisher 344 Rats | 500.00 |
| | TOTAL | $5,000.00 |

Justification

A. Equipment: Dot blot apparatus is required for immobilizing cDNA probes on nitrocellulose for nuclear transciption assays.

B. Ultraviolet Transilluminator: This is an accessory for visualizing DNA or RNA, stained with ethidium bromide in agarose gels.

Polaroid MP-4 Land Camera: This instrument is required for photographing nucleic acids in agarose gels at various stages of the proposed research work. The quotes listed are the lowest available for these items. Currently, none of these instruments are available in the Surgical Research Laboratory or the VA Research Service, where the project will be executed.

Supplies: These represent the supplies for the molecular biology research proposed.

Project Title: Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression in Septic Rat Liver

Principal Investigator: Kalkunte S. Srivenugopal, Ph.D.

## RESEARCH PROPOSAL

### Introduction and Hypothesis

Septic shock is a leading cause of death in Surgical Intensive Care Units, despite the use of specific antibiotics, careful monitoring, and aggressive operative intervention to control the infection (1). A major component of the pathophysiology of bacterial sepsis is a characteristic alteration in carbohydrate metabolism in liver resulting in profound hypoglycemia. This disordered glucose homeostasis, in turn regulates the cell's energy pathways in a negative fashion and makes the patient more suceptible to invading organisms (2-4).

Gluconeogenesis, the synthesis of carbohydrates from non-carbohydrate precursors like alanine, glycerol or lactic acid is now well recognized to be a critical metabolic step in the survival of patients with sepsis. Extensive research from our and other laboratories support the concept of a greatly diminished gluconeogenic capacity in animals with experimental sepsis (3,4). A decreased level of the enzyme activities [e.g., glucose phosphatase, phosphoenolpyruvate carboxykinase (PEPCK)], and the intermediate substrates in gluconeogenesis has been observed consistently in the septic state (5,6). PEPCK catalyzes the first step of gluconeogenesis and regenerates PEP from oxaloacetate. A variety of stimuli such as steroids, hormones, and diet can influence the gene expression of this key enzyme (7). No information is currently available on the expression of PEPCK in septic shock. Understanding the molecular aspects of the regulation of this enzyme and its inducibility in shock can lead to the development of new therapeutic modalities to treat the pathophysiology of sepsis.

The central hypothesis of this proposal is that the expression of PEPCK gene is down-regulated at the transcriptional or posttranscriptional level in rat liver after peritonitis shock. The decreased messenger ribonucleic acid (mRNA) level is postulated to account for the diminished enzyme activity in the septic state. We also hypothesize that steroids like dexamethasone phosphate (DMS) act at the level of gene expression to restore PEPCK levels and thus support gluconeogenesis and enhance survival in rats subjected to peritonitis sepsis.

### Specific Aims

The following are the specific aims of the proposal:

1. To compare and quantitate mRNA levels for PEPCK gene using complementary deoxyribonucleic acid (cDNA) probes in the liver of rats with experimental sepsis (peritonitis) and that of control animals. A correlation of the decreased message level to the reduced enzyme activity will also be performed.

2. To assay the differential rate of transcription of PEPCK in liver cell nuclei, isolated from: (a) control rats, and (b) rats subjected to peritonitis septic shock. These in situ transcription assays are designed to investigate if the gene regulation is at transcriptional level.

3. To examine the mRNA levels and the relative transcription rates in nuclei for PEPCK gene in rat liver from the following experimental groups: (a) control rats, (b) rats subjected to peritonitis, and (c) rats pretreated with steroids [(DMS or methylprednisolone sodium succinate (MPS)] and then subjected to peritonitis.

Methods

I. Rapid Peritonitis Septic Shock Model

4-6 month old Fisher 344 male rats are anaesthesized followed by laparotomy. A 1 cm incision on the avascular surface of cecum is made and ligated; the abdomen is then closed. The devascularized cecum becomes nacrotic and allows the contained stool to spill into the peritoneal cavity. The animal's own gastrointestinal flora causes the sepsis. Rats under these conditions live up to 10 hours after operation. For the gene expression studies, determinations are made at 3, 5, and 9 hours of sepsis.

II. Isolation of Whole Cell RNA from Rat Liver and Northern Blot Analysis to Quantitate mRNA Levels for PEPCK

The liver is minced into small pieces and homogenized in 4M guanidinium thiocyanate solution. The clarified lysate is layered on a cesium chloride cushion in 8 ml centrifuge tubes. After ultracentrifugation at 30,000 rpm for 24 hours, RNA forms a tight pellet at the bottom of the tubes, with DNA and protein in suspension. RNA is further purified by ethanol precipitation. Extreme caution is exercised to avoid ribonuclease (RNase) contamination at all stages.

3-5 µg RNA is fractionated on denaturing agarose gels by electrophoresis in the presence of 2.2 M formaldehyde. RNA from the agarose gels is a 2.2 kb cDNA fragment (for PEPCK) in pBR322 plasmid, isolated by Yoo-Warren, et al, (8) was provided by Dr. R.W. Hanson, Case Western Reserve University, Cleveland, OH. An 800 base pair Sma I restriction fragment of this DNA is nick translated in the presence of $\alpha$-$^{32}$P CTP, deoxyribonuclease (DNase) and DNA polymerase to label the DNA with $^{32}$P. The denatured radioactive cDNA probe (20 x $10^6$ CPM) is hybridized with the RNA on nitrocellulose filters in a hybridization mix for 24 hours at 42°C. The filters are then washed with a low salt buffer (10 mm sodium chloride) and autoradiographed. The visible RNA bands are quantitated by densitometry.

III. Transcription of PEPCK in Isolated Nuclei

This assay helps to distinquish between the transcriptional and posttranscriptional models of gene regulation by the following procedure: (a) Isolation of liver cell nuclei, maintaining the chromatin in its native state; (b) incubation of nuclei in a transcription assay buffer containing Mg++, K+, ribonucleotides and $\alpha$-$^{32}$P-UTP (100 µci) at 37°C for 30 minutes; (c) DNase I treatment of nuclei and purification of RNA by phenol extraction and ethanol precipitation; (d) immobilization of cDNA (10 µg) samples and plasmid DNA samples on nitrocellulose using a slot blot apparatus; (e) equal number of $^{32}$P counts in RNA (purified from

nuclei) from control and septic animals are hybridized with the cDNA on nitrocellulose; and (f) washing of the filters, autoradiography and densitometric quantitation.

IV. Steroid Treatment and Gene Regulation in Sepsis

DMS (1 mg/100 g body weight) or MPS (3 mg/100 g body weight) are administered at the time of peritonitis induction. Northern blot analysis and nuclear transcription studies are performed as described above.

Significance

Since sepsis places increased demands on the host for energy and other substrates for tissue repair and host defence, hepatic gluconeogenesis is critical for the host's adaptation to sepsis, particularly after the depletion of glycogen stores in the initial phase of the pathophysiologic process (3). The identification of agents maximizing gluconeogenic efficacy under these conditions. favors stress resistance and results in increased survival. Some steroid compounds and glucocorticoids appear to support gluconeogenesis in septic state, but their mechanism of action in this process is not clear (9,10).

Glycolysis and gluconeogenesis have most of their enzymes in common. Only at three levels are different enzymes used in gluconeogenesis and they are the points of regulation (Fig. 1). Two crossovers of metabolites occur between these two processes; one at the level of fructose-6-phosphate/fructose 1,6-biphosphate, and the other at the level of pyruvate/phosphoenolpyruvate (11). Studies of carbohydrate metabolism in various septic shock models have generally supported a reduced gluconeogenic capacity (6,12). Such an inhibitory effect can arise from: (1) diminished substrate supply and product inhibition; (2) regulation of key enzyme by allosteric effectors or protein modification; and (3) reduced enzyme levels traceable to its depressed gene expression. Accumulation of the gluconeogenic intermediates like glucose 6-phosphate (G6P), lactic acid, and PEP has been reported in septic shock models (5). The present study concentrates on the third possibility of the altered gene expression being responsible for the lower enzyme levels. The key regulatory role of PEPCK has been well demonstrated as due to the changes of its gene expression in starvation, under the influence of cAMP and other hormones, as well as in the developing fetus (7,13,14). The availability in our laboratory of a cDNA probe for this important enzyme has encouraged us to examine the molecular aspects of its expression and modulation in septic shock. These studies serve as a basis for further evaluation of steroids/hormones and other modulatory factors to improve the efficacy of gluconeogenesis in sepsis at the molecular level. We plan to extend similar studies to look at the gene expression of other regulatory enzymes associated with carbohydrate metabolism in sepsis.

Preliminary Work

Relevant publications from earlier work in our laboratory are attached.

References


1. Wolffe, S.M., Bennett, J.V.: Gram negative rod bacteremia. N. Engl. J. Med. 291:733-737, 1974.

2. Cerra, F.B., Siegel, J.H.. Border, J.R., Wiles, J., McMemamy, R.R. The hepatic failure of sepsis: cellular vs substrate. Surg. 86:409-422, 1979.

3. Schumer, W.. Overall cell metabolism, molecular and cellular aspects of shock and trauma. Alan R. Liss Inc., New York: p 1-19, 1983.

4. Miller, S.I., Wallace, R.J., Musher, D.M., Septimus, E.J., Kohl, S., Baughn, R.E. Hypoglycemia as a manifestation of sepsis. Am. J. Med. 68:549-654, 1980.

5. Kuttner, R.E., Holtzman, S.F., Schumer, W. A time course study of hepatic glycolytic intermediates in endotoxemic and septic rats and mice. Adv. Shock Res. 4:73-85, 1980.

6. Apantaku, F., Ebata, T., Kuttner, R., Schumer W. Effect of peritonitis on key glucoregulatory enzymes in rat liver. Circ Shock 13:269-273, 1984.

7. Goodridge, A.G. Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. Ann. Rev. Nutr. 7:157-185, 1987.

8. Yoo-Warren, M., Cimbala, M.A., Felz, J.E. Monahan, J.P. Hanson, R.W. Identification of a DNA clone to phosphoenolpyruvate carboxykinase (GTP) from rat cytosol. J. Biol. Chem. 256:10224-10227, 1981.

9. Kuttner, R., Apantaku, F., Schumer, W. Glucocorticoid effect on glycolytic intermediate in septic rat heart. Adv. Shock Res. 5:103-111, 1981.

10. Schumer, W. Pathophysiology and treatment of septic shock. Am. J. Emerg. Med. 2:74-77, 1984.

11. Hers, H.G., Hue, L. Gluconeogenesis and related aspects of glycolysis. Ann. Rev. Biochem. 52:617-653, 1983.

12. Knowles, R.G., MCabe, J.P., Beevers, S.J., Pogson, C.I. The characteristics and site of inhibition of gluconeogenesis in rat liver cells by bacterial endotoxin. Biochem. J. 242:721-728, 1987.

13. Pilkis, S.J., El-maghrabi, R. Hormonal regulation of hepatic gluconeogenesis and glycolysis. Ann. Rev. Biochem. 57:755-783, 1988.

14. Lyonnet, S., Coupe, C., Girad, J., Kahn, A., Munnich, A. In Vivo regulation of glycolytic and gluconeogenic enzyme gene expression in new born rat liver. J. Clin. Invest. 81:1682-1689, 1987.

VETERANS ADMINISTRATION MEDICAL CENTER
North Chicago, Illinois

## Application For Use of Radionuclides in Research Studies

INSTRUCTIONS: Please TYPE all information and forward to the Radiation Safety Committee, mail code 115

1. Principal Investigator: Kalkunte Srivenugopal, Ph.D.

2. Department: UHS/CMS Department of Surgery

3. Title of Research Project: Regulation of Phosphoenolpyruvate Carboxynase Expression in Septic Rat Liver

4. VA Project Number: N/A

5. Application Type: N/A

( X ) Initial ( ) Renewal ( ) Amendment

6.

| TRAINING AND EXPERIENCE OF AUTHORIZED USER (Use Supplemental Sheets if Necessary) | | | | |
|---|---|---|---|---|
| TYPE OF TRAINING | WHERE TRAINED | DURATION TRAINING | ON THE JOB | FORMAL COURSE |
| a. Principles & Practices of radiation protection | Univ. Washington Seattle | 7 years | X Yes/No | X Yes/No |
| b. Radioactivity measurement standardization & monitoring techniques & instruments | " | " | X Yes/No | X Yes/No |
| c. Mathematics & calculations basic to the use & measurement of radioactivity | " | " | X Yes/No | X Yes/No |
| d. Biological effects of radiation | " | " | X Yes/No | X Yes/No |

EXPERIENCE WITH RADIATION (Actual use of radioisotopes or equivalent experience).

| ISOTOPE | MAXIMUM AMOUNT | WHERE EXPERIENCE WAS GAINED | DURATION OF EXPERIENCE | TYPE OF USE |
|---|---|---|---|---|
| $^{14}C$ | 100 µCi | Indian Inst. Sci. Bangalore, India | 2 years | Research |
| $^{3}H$ | 250 µCi | " | 3 years | Research |
| $^{32}P$ | 1 mCi | Univ. Washington Seattle | 2 years | Research |

*) Circle one
VAF 10-250(556)
April 1986

CONTROL NO. 8795

7. Names and experience of all personnel involved in handling radioisotopes.

| NAME | TYPE OF TRAINING | LOCATION | ON THE JOB | FORMAL |
|---|---|---|---|---|
| N/A | N/A | N/A | N/A | N/A |

8. Location of use: Building 1, Room(s) 236

a. Radioisotope Storage — Bldg. 1, Room 236

b. General Laboratories — same as above

c. Human Administration — None

d. Animal Administration — None

e. Counting Room — Bldg. 1, Room 250

f. Animal Storage Area — N/A

9. Radioisotopes to be used:

a. Radioisotopes — $^{14}C$, $^{3}H$, and $^{32}P$

b. Chemical and/or Physical Form Purchased — mostly liquid

c. Sterile — yes

FDA Approved — yes

d. Supplier — New England Nuclear, Inc.

e. Maximum Activity (in millicuries) to be Ordered at One Time — up to 2 mCi

f. Maximum Activity (in millicuries) to be Possessed at One Time — up yo 2 mCi

g. Size of Human and/or Animal Dose (in millicuries) — N/A

CONTROL NO. 87935

h. Maximum Number of Doses to be Administered to Each Human and/or Animal: N/A

i. Route of Radioisotope Administration: N/A

j. Name of Physician to Administer Radioisotope to Humans: N/A

k. Maximum Activity (millicuries) to be used In-Vitro: 10 µCi to 100 µCi

10. Patient and/or Animal Data:

a. Type of Patient or Species of Animal: N/A

b. Total Number of Human Subjects: N/A

c. Special Preparation of Humans and/or Animals (Surgical Procedures, Etc...): N/A

d. Critical Organ: N/A

e. Methods to be Used in Counting Samples: N/A

11. Radiation Safety Precedures (Monitoring, Survey Meters, Special Pipetting devices, etc...)

Usual radiation safety procedures involving immediate cleanup, safe storage of waste, monitoring of work area with Geiger counter (for $^{32}P$) will be undertaken.

CONTROL NO. 87985

12. Disposition of Radioactive Waste (Including Unused Material)

The radioactive waste will be disposed in solid form, according to the radiation safety guidelines.

13. Attach a description of the General Purpose and Investigative Protocol.

$^{14}C$ and $^{3}P$ isotopes will be used in in vitro reactions and metabolic studies of septic shock and cancer research. $^{32}P$ nucleotides will be used to label DNA in molecular biology studies. (Copy of research project is attached.)

Karl Knute Strömquist

Signature of Principal Investigator

Date February 24, 1989

CONTROL NO. 87925

Principal Investigator

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed, beginning with the Principal Investigator.

| Name | Title | Birthdate (Mo.Day,Yr.) |
|---|---|---|
| Kalkunte S. Srivenugopal | Ph.D. | June 7, 1955 |

Education (Begin with baccalurate training and included postdoctoral

| Institution and Location | Degree | Year Conferred | Field of Study |
|---|---|---|---|
| Bangalore University<br>Bangalore, India | B.S. (honors) | 1973 | Botany, Chemistry<br>Zoology |
| Bangalore University<br>Bangalore, India | M.S. | 1975 | Botany |
| India Institute of Science<br>Bangalore, India | Ph.D. | 1980 | Biochemistry |

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications.

Previous Employment

1980-1981 Teaching Assistant, Department of Biochemistry, Indian Institute of Science, Bangalore, India

1981-1987 Postdoctoral Fellow, Lymphocyte Gene Expression and Polyamine Research Laboratory, Department of Biochemistry, University of Washington School of Medicine, Seattle, Washington

1987-1988 Senior Fellow, Northwest Neuro-Oncology Laboratory Department of Neurological Surgery, University of Washington School of Medicine, Seattle, Washington

1988-present Assistant Professor, Division of Surgical Research, Department of Surgery, University of Health Sciences/ The Chicago Medical School, North Chicago, Illinois

Awards and Honors Professor Giri Memorial Medal for the best Ph.D. thesis, Biochemical Society, Indian Institute of Science, Bangalore

Publications

1. Srivenugopal, KS, Adiga, PR. The opiate receptor and opioid peptides (a review). J Indian Inst Sci 59:187-228, 1977.

2. Srivenugopal, KS, Adiga, PR. Coexistence of two pathways of spermidine biosynthesis in lathyrus sativus seedlings. FEBS Lett 112:260-264, 1980.

Publications (Continued)


3. Srivenugopal, KS, Adiga, PR. Artifactual staining of proteins on polyacrylamide gels by nitrobluetetrazolium chloride and phenazine methosulphate. Anal Biochem 101:215-220, 1980.

4. Srivenugopal, KS, Adiga, PR. A simple procedure for purification of n-carbamylputrescine: application to assays of putrescine transcarbamylase and agmatine iminohydrolase activities. Anal Biochem 104:404-444, 1980.

5. Srivenugopal, KS, Adiga, PR. Partial purification of properties of a transamidinase from lathyrus sativus seedlings: involvement in homarginine metabolism and amine interconversions. Biochem J 189:553-560, 1980.

6. Srivenugopal, KS, Adiga, PR. Enzymic synthesis of symhomospermidine in lathyrus sativus seedlings. Biochem J 190: 461-464, 1980.

7. Srivenugopal, KS, Adiga, PR. Enzymic conversion of agmatine to putrescine in lathyrus sativus seedlings: purification and properties of a multifunctional enzyme (putrescine synthase). J. Biol. Chem 256:9532-9541, 1981.

8. Srivenugopal, KS, Adiga, PR. Putrescine synthase from lathyrus sativus (grass pea) seedlings. Meth Enzymol 94:333-339, 1983.

9. Srivenugopal, KS, Adiga, PR. Preparation and purification of n-Carbamylputrescine and (ureido-$^{14}$C)-N-carbamoyl putrescine. Meth Enzymol 94:429-430, 1983.

10. Srivenugopal, KS, Lockshon, D, Morris, DR. Escherichia coli DNA topoisomerase III: purification and characterization of a new type I enzyme. Biochemistry 23:1899-1906, 1984.

11. Srivenugopal, KS, Morris, DR. Differential modulation by spermidine of reactions catalyzed by type I prokaryotic and eukaryotic topoisomerases. Biochemistry 24:4766-4771, 1985.

12. Wemmer, DE, Srivenugopal, KS, Reid, BR, Morris, DR. Nuclear magnetic resonance studies of polyamine binding to a defined DNA sequence. J Mol Biol 185:457-459, 1985.

13. Srivenugopal, KS, Morris, DR. Modulation of the relaxing activity of Escherichia coli topoisomerase I by single-stranded DNA binding proteins. Biochem Biophys Res Commun 137:795-800, 1986.

14. Srivenugopal, KS, Wemmer, DE, Morris, DR. Aggregation of DNA by spermidine analogs: enzymatic and structural studies. Nucleic Acids Res 15:2563-2580, 1987.

15. Morris, DR, Abrahamsen, MS, Srivenugopal, KS, Rabinovitch, PS, Kuepfer, CA White, MW. Roles of calmodulin and protein kinase c in regulation of the c-myc and ornithine decarboxylase genes during mitogenic activation of bovine t-lymphocytes and mouse 3T3 cells. (Submitted to J Biochem).

16. Srivenugopal, KS, Que, B Ali-osman, F. Differential effects of polyamines and inorganic cations on 1,3-bis (2-chloroethyl) 1-nitrosourea (BCNU) induced strand breaks and interstrand cross-linking in col $E_1$ plasmid DNA. (Submitted to Biochem Pharmacol).

17. Srivenugopal, KS, Ali-osman, F. Differential inducation of DNA interstrand crosslinks and single strand breaks by 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) in two human glioma cell lines of varying BCNU resistance. (Submitted to Anticancer Research).

EXPERIENCE WITH RADIATION (Actual use of radioisotopes or equivalent experience).

| ISOTOPE | MAXIMUM AMOUNT | WHERE EXPERIENCE WAS GAINED | DURATION OF EXPERIENCE | TYPE OF USE |
|---|---|---|---|---|
| $^{14}C$ | 100 µCi | Indian Inst. Sci. Bangalore, India | 2 years | Research |
| $^{3}H$ | 250 µCi | " | 3 years | Research |
| $^{32}P$ | 1 mCi | Univ. Washington Seattle | 2 years | Research |

TRAINING AND EXPERIENCE OF AUTHORIZED USER

| TYPE OF TRAINING | WHERE TRAINED | DURATION TRAINING | ON THE FORMAL JOB | COURSE |
|---|---|---|---|---|
| a. Principles & Practices of radiation protection | Univ. Washington Seattle | 7 years | X Yes/No | X Yes/No |
| b. Radioactivity measurement standardization & monitoring techniques & instruments | " | " | X Yes/No | X Yes/No |
| c. Mathematics & calculations basic to the use & measurement of radioactivity | " | " | X Yes/No | X Yes/No |
| d. Biological effects of radiation | " | " | X Yes/No | X Yes/No |

Veterans Administration

# Memorandum

Date: March 7, 1989

From: Chairman, Radiation Safety Committee (115)

Subj: Research Proposal

To: Dr. Kalkunte Srivengopal (151)

1. This is to inform you that your project entitled , "Regulation of Phosphoenolpyruvate Carboxynase" was approved by the Radiation Safety Committee.

2. From the aspect of radiation safety, you may begin your project.

GREGORY A. GERGANS, M.D.

cc:
Medical Research Service (151)
Radiation Safety Officer (11B)

Medical Center North Chicago IL 60064

Veterans Administration

SEP 19 1989

In Reply Refer To: 556/11B

Regional Director (10BA4/115)
Great Lakes Region
Veterans Affairs Central Office
810 Vermont Avenue, NW
Washington, DC 20420

SUBJ: Amendment of U.S. Nuclear Regulatory Commission license number 12-10057-04.

1. Enclosed is a request addressed to the United States Nuclear Regulatory Commission for an amendment to our license number 12-10057-04.

2. This amendment will allow us to add one authorized user of radioactive licensed material for laboratory research including animal studies.

Leonard C. Rogers
Medical Center Director

Enclosures: 4

cc:
Regional Director (10BA4)
Ann Arbor, MI 48106

4/24/89

JAMES W. FLETCHER, M.D.
Director, Nuclear Medicine Service (115)
Veterans Administration
Washington, DC 20420

SEP 25 1989

FEE EXEMPT

CONTROL NO. 87985

Medical Center North Chicago IL 60064

Veterans Administration

SEP 1 [illegible]

In Reply Refer To: 556/11B

United States Nuclear Regulatory Commission
Material Licensing Section
799 Roosevelt Road
Glen Ellyn, IL 60137

Gentlemen:

Please amend our by-product material license number 12-10057-04, to include Georg F. Springer, M.D. as an authorized user of licensed material for laboratory research, including animal studies.

Enclosed is his training and experience in handling radionuclides, the type of use, where the experience was gained, and the duration of each experience.

Dr. Springer has submitted a research proposal which was approved by our Radiation Safety Committee.

Sincerely yours,

Leonard C. Rogers
Medical Center Director

Enclosures:
Research Proposal
Curriculum Vitae
Approval of the Radiation Safety Committee

NORTH CHICAGO, ILLINOIS

2202

Application For Use of Radionuclides in Research Studies

Karen Ridge

INSTRUCTIONS: Please TYPE all information and forward to the Radiation Safety Committee, mail code 115.

1. Principle Investigator: Georg F. Springer, M.D.
2. Department: CMS Dept. of Immunology and Microbiology, Dept. of Surgery
3. Title of Research Project: Carcinoma Pathogenesis: Role of T and Tn Antigen
4. VA Project Number:
5. Application Type:

(x) Initial ( ) Renewal ( ) Amendment

6.

| TRAINING AND EXPERIENCE OF AUTHORIZED USER (Use Supplemental Sheets if Necessary) | | | | |
|---|---|---|---|---|
| TYPE OF TRAINING | WHERE TRAINED | DURATION OF TRAINING | ON THE JOB | FORMAL COURSE |
| a. Principles & practices of radiation protection | Chicago Medical School/UHS | 6 mo. | (Yes) / No *) | (Yes) / No |
| b. Radioactivity measurement standardization & monitoring techniques & instruments | CMS | 6 mo. | (Yes) / No | Yes / (No) |
| c. Mathematics & calculations basic to the use & measurement of radioactivity | CMS/UHS and Illinois Benedictine College | 6 mo. | (Yes) / (No) | (Yes) / No |
| d. Biological effects of radiation | CMS | 6 mo. | (Yes) / No | Yes / (No) |

EXPERIENCE WITH RADIATION (Actual use of radioisotopes or equivalent experience.)

| ISOTOPE | MAXIMUM AMOUNT | WHERE EXPERIENCE WAS GAINED | DURATION OF EXPERIENCE | TYPE OF USE |
|---|---|---|---|---|
| $^{86}Rb$ | 5 mCi | CMS | 1 1/2 yrs. | in vitro |
| $^{45}Ca$ | 2 mCi | CMS | 1 1/2 yrs. | in vitro |
| $^{3}H$ | 1 mCi | CMS | 1 1/2 yrs. | in vitro |
| $^{51}Cr$ | 2 mCi | Evanston Hospital | 6 mos. | in vitro |

CONTROL NO. 87985

## Application For Use of Radionuclides in Research Studies

INSTRUCTIONS: Please TYPE all information and forward to the Radiation Safety Committee, mail code 115.

1. Principle Investigator: Georg F. Springer, M.D.
2. Department: CMS Dept. of Immunology and Microbiology, Dept. of Surgery
3. Title of Research Project: Carcinoma Pathogenesis: Role of T and Tn Antigens
4. VA Project Number: _____
5. Application Type: _____

   ( x) Initial ( ) Renewal ( ) Amendment

6.

| TRAINING AND EXPERIENCE OF AUTHORIZED USER (Use Supplemental Sheets if Necessary) | | | | |
|---|---|---|---|---|
| TYPE OF TRAINING | WHERE TRAINED | DURATION OF TRAINING | ON THE JOB *) | FORMAL COURSE |
| a. Principles & practices of radiation protection | Ohio State University | 10 wks. | (Yes) / No | (Yes) / No |
| b. Radioactivity measurement standardization & monitoring techniques & instruments | Ohio State University | 10 wks. | (Yes) / No | (Yes) / No |
| c. Mathematics & calculations basic to the use & measurement of radioactivity | Ohio State University | 10 wks. | (Yes) / No | (Yes) / No |
| d. Biological effects of radiation | Ohio State University | 10 wks. | (Yes) / No | (Yes) / No |

EXPERIENCE WITH RADIATION (Actual use of radioisotopes or equivalent experience.)

| ISOTOPE | MAXIMUM AMOUNT | WHERE EXPERIENCE WAS GAINED | DURATION OF EXPERIENCE | TYPE OF USE |
|---|---|---|---|---|
| $^{3}H$ | 10 µCi | Ohio State University | 6 yrs. | in vitro |
| $^{14}C$ | 250 µCi | Ohio State University | 6 yrs. | in vitro |
| $^{125}I$ | 1 mCi | Ohio State University | 3 yrs. | in vitro |
| $^{51}Cr$ | 40 mCi | Ohio State University | 1 yr. | in vitro |
| $^{51}Cr$ | 2 mCi | Evanston Hospital | 6 mo. | in vitro |

*) Circle one

7. Names and experience of all personnel involved in handling radioisotopes

| NAME | TYPE OF TRAINING | LOCATION | ON THE JOB YES/NO | FORMAL YES/NO |
|---|---|---|---|---|
| Karen M. Ridge, B.S. | See question #6 | CMS | Yes | Yes |
| Jeffrey Smoot, Ph.D. | See question #6 | CMS | Yes | Yes |

8. Location of use: Building 1, Room(s) 129

a. Radioisotope Storage Room 129

b. General Laboratories Room 129

c. Human Administration N/A

d. Animal Administration N/A

e. Counting Room Room 124

f. Animal Storage Area N/A

9. Radioisotopes to be used:

a. Radioisotopes Chromium - 51

b. Chemical and/or Physical Form Purchased Sodium chomate in sterile 0.9% sodium chloride solution

C. Sterile Yes

FDA Approved

d. Supplier Amersham Corporation

e. Maximum Activity (in millicuries) to be Ordered at One Time 1 mCi

f. Maximum Activity (in millicuries) to be Possessed at Any One Time ~~4 mCi~~ 2 mCi

g. Size of Human and/or Animal Dose (in millicuries) Does not apply

CONTROL NO. 87935

h. Maximum Number of Doses to be Administered to Each Human and/or Animal: N/A

i. Route of Radioisotope Administration: N/A

j. Name of Physician to Administer Radioisotope to Humans: N/A

k. Maximum Activity (millicuries) to be Used In-Vitro: N/A

9. Patient and/or Animal Data:

a. Type of Patient or Species of Animal: N/A

b. Total Number of Human Subjects: N/A

Animal Subjects: N/A

c. Special Preparation of Humans and/or Animals (Surgical Procedures, Etc...): N/A

d. Critical Organ: N/A

e. Methods to be Used in Counting Samples: N/A

10. Radiation Safety Procedures (Monitoring, Survey Meters, Special Pipetting devices, etc...)

See investigative protocol

11. Disposition of Radioactive Waste (Including Unused Material)

See investigative protocol

12. Attach a description of the general purpose and investigative protocol.

Guy Springer M.D.

Signature of Principal Investigator

Date: 6.15.89

CONTROL NO. 87985

Application For Proposed Use of $^{51}Cr$ In The Investigation of Cell-Cell Adhesion

I. Proposed Use and Objectives:

Sodium [$^{51}Cr$] - chromate will be incorporated into cells of normal, benign, or malignant phenotype. The isotopically labeled cells will then be used to probe the qualitative and/or quantitative extent of their adherence to experimentally prepared monolayers of non-radiolabeled cells, both homotypic and heterotypic to the labeled cells. This experimentation will require the use of up to 4 mCi every second week.

II. Description of Procedure to be used:

The stock container of aqueous sodium [$^{51}Cr$] - chromate will be stored in a lead containment carrier. On the day of experimentation, a 50-100 μCi aliquot will be withdrawn and placed into a disposable plastic culture tube containing $10^4$-$10^6$ cells suspended in a physiological buffer. Following a 1 hr. incubation, the labeled cells will be washed free of unincorporated $^{51}Cr$ and then centrifuged onto unlabeled monolayers established in PVC microtiter plates for assessment of adherence.

III. Waste Handling and Disposal:

Solid waste (gloves, pipets tips, culture tubes) and titer plates (after air drying in a hood) will be placed into a container so designated for such disposal. Bulk liquids (cell washings containing ≥95% of $^{51}Cr$ unincorporated) will be placed into an appropriate container provided or approved by the Radiation Safety Office. Periodic waste pick-up will be scheduled through RSO to maintain possession limits within the usage limit above.

IV. Monitoring of Laboratory, Equipment and Personnel:

The experimentation area and centrifuge to be monitored by smear wipe. Areas exhibiting radiation in excess of 200 DPM/100 $cm^2$ are subject to decontamination with subsequent wipes to fall below the 200 DPM/100 $cm^2$ limit. Personnel are to wear film badges to be submitted for exposure dosimetry.

V. Safety Analysis:

1. Chemical:
   There are no inherent dangers. As supplied by the manufacturer, the aqueous solutions of sodium chromate are neither flammable nor explosive.

2. Biological:
   The critical organ(s) for $^{51}Cr$ is total body wherein the biological half-life is 616 days. The maximum permissible body burden is 800 μCi.

3. Radiological:
   $^{51}Cr$ has a physical half-life of 27.7 days. The major source of emitted energy consists of a gamma particle of 0.32 MeV. During a single experiment the greatest exposure to gamma radiation would be on withdrawal of an aliquot from the stock container. At a distance of one foot, the exposure presented by a 4 mCi stock vial of $^{51}Cr$ would not exceed:

$$\frac{1.92 \text{ mR}}{\text{hr-mCi}} \times 4 \text{ mCi} = 7.68 \frac{\text{mR}}{\text{hr}}$$

The worst feasible accident would involve breakage of the stock container. In this event, contaminated personnel will be restricted to the immediate area for survey and decontamination. Non-essential personnel will be denied access. Radiation Safety will be advised of the spill and their personnel will direct the proper disposal procedure.

Dr. Georg Springer Labs
Karen M. Ridge

## A. Materials

a1) Surgical Specimens. From Dr. T.A. Victor (grant consultant and Chief, Dept. Surgical Pathology, Evanston Hospital) we will obtain standard paraffin blocks for IHC study of most of the needed tissue. In addition, tissues will be taken immediately after their surgical excision and where indicated up to 15 min. thereafter, provided that there remains more than sufficient appropriate material for pathological study.

Breast CA, benign and normal breast glandular tissues: A spetrum of breast lesions will be collected separately under sterile conditions as above and kept in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum [FBS] (cf. 42,43). In decreasing order of malignancy they will range from invasive Stage I (ductal, tubular, lobular), microinvasive and Tis (in situ) breast CAs to premalignant, proliferative ductal and lobular atypical hyperplasia, papillomatous sclerosing adenosis to strictly benign lesions and healthy breast glandular structures (104,105), including those from reductive mammoplasty.

a2) Tissue Culture Cell Lines of Human AdenoCAs and Controls. We maintain established CA lines in suspension cultures in RPMI 1640 plus 10% FBS: DU 4475 (EG&G Mason, Boston, MA), from a metastatic skin nodule of a ductal breast CA; healthy colon mucosa-derived epthelial-like HCMC (ATCC, CCL 239); healthy urinary bladder epithelium line HS 767B (53) one line of healthy breast epithelial cells is from Dr. A. Hackett (cf. 29,31); HTB 23, from a ductal breast CA metastasis; and NCI-H69, from a metastasis of a small-cell lung CA. The last two lines are from the ATCC, both from pleural effusions. Line DU 4475 cells contain more Tn than T Ag. We have grown ~ 1 x $10^{11}$ CA cells of this line with high Tn and some T activity from which we have isolated and immunologically and chemically characterized Tn glycoprotein (16).

These cells will serve not only as a source of Tn-specific EPs and haptens in adhesion-inhibition experiments but also as a source of CA cell-surface ligands involved in Tn-specific CA to CA adhesion. We maintain cryopreserved a number of additional breast CA lines, defined by the ATCC: HTB 20, HTB 121 and HTB 131, isolated from primary lesions and the only ones that hold any promise of growing, with prodding, in suspension culture (needed for mass production).

We will grow in short-term cultures human breast CA, benign and normal cells derived from surgical specimens. Techniques: We will obtain and grow all cells according to standard procedures, including those of A. Hackett and her colleagues (70,106). Both malignant and healthy cells will also include established lines. The Peralta group has already sent a healthy breast epithelial cell line to us. We will visit this group (Peralta Cancer Research Institute, Oakland, CA) to learn practical details of their successful techniques for growing normal breast epithelia, which they grow with about 1½ times greater success than corresponding CA cells (71).

a3) Human Peripheral Blood Collection. For anti-Tn Ab determinations, blood is collected under standard conditions, allowed to clot at room temperature, and kept overnight at ~ 4°C. The serum is divided into aliquots and stored at -80°C or in liquid nitrogen, because long-term storage at temperatures above -30°C and repeated freezing and thawing damages anti-Tn Abs (unpublished).

a4) Large-Scale Preparation of RBC-derived Tn Ag. We have previously prepared small quantities of RBC-Tn Ag (77,78) by a tedious procedure unsuitable for preparative work because of difficulty in specific removal of the β1-3 linked Gal from the T hapten. Recently, through the kindness of Drs. Distler and Jourdian, we obtained a large quantity of bovine testis galactosidase (85), which readily and specificially hydrolyzes the β1-3

ploidy and therefore CA (71).

A major advantage of the CAS system compared to flow systems is the former's ability to recognize tumor cell morphology, Ags, and DNA content and the certainty that the measurements are not affected by inflammatory, necrotic or stromal elements.

Since our main interest is to define the role of Tn in CA pathogenesis, we will determine Tn EP densities and distribution in relation to T EPs on and in highly differentiated CAs and compare them with density and distribution in anaplastic CAs and premalignant tissue. We will also compare expression of cell-surface Tn on early breast tumors that have different malignant potentials [ductal versus lobular and tubular breast CA, and check for the possible presence of Tn and T in atypical & hyperplastic-proliferative benign lesions versus truly benign and normal epithelia (105,129,130)].

b2) Study of the Role of Human Breast CA Cell-Surface Tn Ag in Adhesion of CA Cells to Benign and Normal Breast Epithelia (and to Each Other).

Our goal here is to study primary, preferably early, CAs obtained at surgery and up to 15 min. thereafter, to assess CA interaction with its microenvironment, which consists of mostly benign and normal epithelia. Such studies will be done on fixed surgical specimens by IHC and also by automated, quantitative cell image analysis [see 2D.B.b1)]. However, histology does not always allow insight into dynamic processes. Therefore, we will investigate the interaction of live, early CA cells with their microenvironment, by means of the adhesion experiments proposed here: These studies on early CA should permit determination of adhesive forces between CA and adjoining epithelia, in fact, our preliminary studies [see 2C.c)] show that measurement of centrifugal shear forces needed to separate live malignant cells is feasible, and it has been shown earlier by others for nonmalignant epithelioid cells. The specific cell-cell adhesion has been subdivided into recognition of complementary receptors and ligands on apposing surfaces of adjoining cells and reversible adhesion, which eventually leads to irreversible adhesion (91,92,131,132).

We will study in vitro adhesion between CA and nonCA epithelial cells: the preconditions and sequential steps from specific recognition ⟶ reversible adhesion ⟶ irreversible adhesion. Elucidation of the basis of these early interactions between apposing cell surfaces of primary CA and nonmalignant epithelia should enable us to define some of the intrinsic factors that lead to microinvasion by primary CAs followed by gross tissue invasion.

Our preliminary studies have clearly shown the importance of Tn in cancer cell adhesion to healthy cells and revealed that the adhesion process itself can be produced and specifically inhibited [see 2C.d)]. The forces that underlie adhesion of primary ductal and lobular breast CA cells to benign diseased and healthy breast epithelia will be studied quantitatively. Wherever possible, malignant, benign and normal tissues will be from the same patient.

(i) Commercially available in vitro tissue culture-grown CA cell lines and three presumably normal epithelial cell lines to be used are listed in 2D.A.a2). All procedures will be carried out under sterile conditions. Preparation of single-cell suspensions from tissues obtained at surgery will be patterned after the standard techniques of others (30,70,71). Immediately after surgical removal of breast tissue, those live breast glandular tissues not needed by the pathologist will be dissected away from skin and fatty areas, minced into ~ 1 $mm^3$ cubes, digested with dispase (Boehringer Mannheim, Indianapolis, IN; Calbiochem, San Diego, CA). This highly effective, sterile protease and collagenase mixture does not interfere with cell growth [see also (133)]. The digest will be filtered through a screen (100 pylar equiv. mesh) and yields >60% live cells as determined by trypan blue exclusion. A portion of the recovered cells will then be prepared for study and the rest, quantity permitting, for culturing and cyropreservation. The cells to be used for immediate study will be maintained alive for 24-48 hrs. in Eagle's minimum essential medium [MEM] (GIBCO) containing 10% fetal bovine serum [FBS] (Hyclone Labs., Logan, UT) in a humidified air atmosphere, containing 5% $CO_2$, at 37°C, to restore cell membrane glycoproteins and integrity. Live cells will then be separated by gradient centrifugation using Ficoll-Paque or Percoll (Pharmacia, Piscataway, NJ) and used for experimentation.

Cells from surgical specimens will be grown as described in (16). FBS will be reduced to 5% in suspension cultures (attempts will not always be successful, but have been productive several times in preliminary studies). Single-cell-suspension growth will be in Wheaton Magnaflex flasks with Wheaton "Micro-stir" slow speed magnetic stirrers (Wheaton Instr., Millville, NJ). Growth medium (RPMI 1640 + 5 to 10% FBS) and conditions to initiate and maintain growth (propagation) will be those previously described (16). At this time, we do not plan to experiment with one of the several commercial protein-free media.

(ii) Our technical approach to the adhesion studies incorporates major procedures used by others in nonCA systems (91,93,134), which have proved effective in our preliminary studies. Specific adhesion and its inhibition will be based on our own work (19,23,24). We also have established contact with Dr. D.R. McClay, Duke University (91) and obtained useful practical modifications of his adhesion protocols.

The principle of the proposed binding assay is outlined in Fig. 1. A Damon/IEC centrifuge with temperature adjustable to constancy between 0°C to 39°C will be used throughout with rotor for horizontal centrifugation of plate carriers up to 2,350g (3,400 rpm). All binding assays will be done in quadruplicate. Flat-bottom polyvinyl chloride (PVC) plates (tissue-culture treated), with 48 or 96 wells (Dynatech, Alexandria, VA), will be used. Although our preliminary results using a saturated waxbean agglutinin solution (93) were satisfactory (Springer, G.F. & Gu, J., to be published), we have obtained more reproducible results with the modified McClay procedure (91 and modifications by M.A. Alliegro, C.A. Burdsal, M.M. Lotz, and D.R. McClay, 1988, unpublished).

Fifty µl of 1 mg/ml poly-D-lysine (PDL) [Sigma P-1024] in carbonate buffer, pH 9.6, were added to each well and the wells incubated for 2 hrs. at room temp. Washing 2x by gentle aspiration with carbonate buffer (100 µl/well) followed. Suspension of cells for the bottom monolayer is adjusted to a cell concentration that resulted in a monolayer at 100 µl/well. This suspension is added (100 µl/well) to PDL precoated wells. Cells are then centrifuged onto plate bottoms at 4°C and 180g for 5 min (see Fig. 1.A). Using an inverted microscope, the monolayer is verified; if confluent, cells are incubated with 100 µl medium for 1 hr. at 37°C in a water-saturated atmosphere containing 5% $CO_2$. The medium is aspirated from each well and the wells incubated with 100-150 µl 10% BSA (blocking solution) for 1 hr. at room temp. This step blocks any remaining exposed plastic in the reaction area of the wells. The BSA solution is then replaced by the medium. The monolayer cells, after 1 hr. of incubation on PDL-coated wells at 37°C resist dislodgement from the plastic by forces of >3,000g, regardless of whether benign or malignant cells are used.

Optimal conditions for preparation of initial monolayers have been established with our abundant suspension-grown DU 4475 breast CA cells and small-cell lung CA NCI-H69 as models, and also with colon epithelial line HCMC (ATCC CCL 239). These "substrate" monolayer cells will also originate from "normal" human mammary epithelial cells of surgical specimens after short-term culturing [see 2D.A.a1) and (70)]. Long-term CA cell lines will be used as substratum when normal or benign breast epithelial cells are used as probe cells, and also, when CA — CA adhesion is studied.

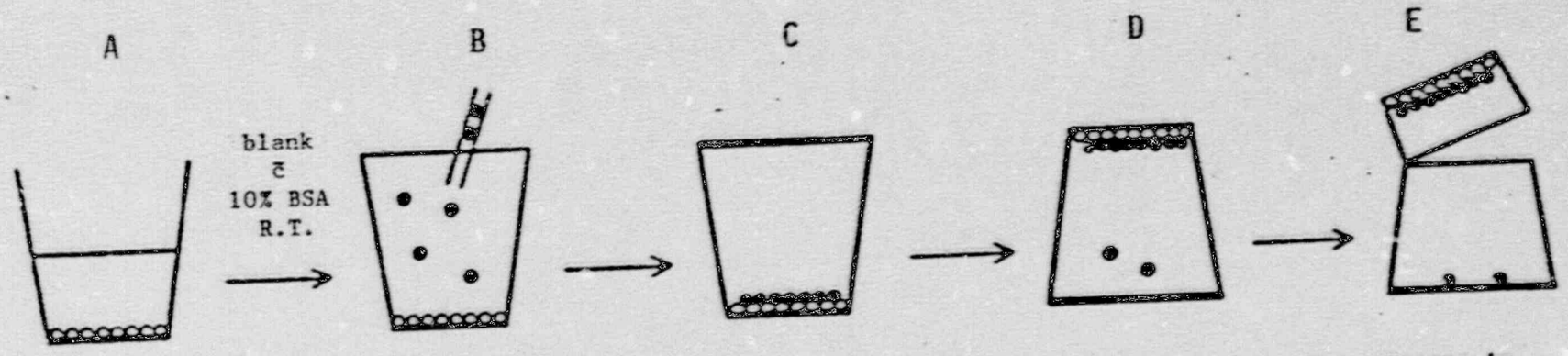
[$^{51}$Cr]-labeled (135) epithelial cells, as probe cells will then be added on top of the first monolayer several hrs. after the latter's formation (Fig. 1.B). The wells are sealed after probe cell addition by rolling on an adhesive microtiter plate sealer (Dynatech). The precautions listed in (91) plus additional ones communicated by Dr. McClay will be taken. The probe cells will be centrifuged onto the monolayer (10 min. at 10g at the desired adhesion temperature) and allowed to adhere for 90 min. (Fig. 1.C). The experiments will be performed at 37°C and at 4°C. In early experiments, the probe cells will consist of cultured breast CA single-cell suspensions [see listing in 2D.A.a2)].

[redacted]

After formation of the second layer, the plates will be inverted and centrifuged so that centrifugal force now tends to pull the probe cells from the monolayer (Fig. 1D). The original monolayer is not dislodged by forces 3-fold greater than the maximum shear-

Fig. 1: SCHEMATIC DIAGRAM OF CELL BINDING ASSAY*

Based on ref. 91




*See text.

Blood group O RBC tested in this assay did not adhere to test or O RBC monolayers at 1g dislodgment force.

ing forces used in our experiments. These forces also did not seem to damage the cells (trypan blue exclusion). The plates will then be placed, still sealed and inverted, into isopentane/dry ice in a "Histobath" (Neslab, Portsmouth, NH) to freeze their contents; the temperature approaches that of liquid $N_2$ and is conveyed by conduction, thus avoiding undesirable crystal formation in the samples. The bottoms of the frozen wells containing the monolayers are clipped off with a pet toenail clipper cutting at exactly 3 mm above bottom. [redacted] The [$^{51}$Cr]-labeled cells are assayed in a γ-counter.

Background corrections, controls and standard will be comparable to those used in ref. 91 plus addendum.

We have been able to prevent specific adhesion of CA to healthy epithelioid or CA monolayers by picogram quantities of both Tn and T glycoproteins but not by 8- to 10-fold larger amounts of similar glycoproteins carrying no Tn or T EPs. The systems proposed in this section will be evaluated for adhesion specificity using RBC-Tn, CA-Tn and -T, and other well-defined Tn- and T-active and -inactive soluble (glyco)proteins and (glyco)peptides as inhibitors of specific cell ligands. Free oligo- and monosaccharides will also be used.—Blocking of CA cell adhesion will be accomplished by preincubation of the probe cells with anti-Tn Fab fragments obtained from affinity purified human polyclonal and rodent monoclonal anti-Tn Abs [see 2D.A.a7)]. These studies will assess what role the Tn and T EPs of the probe cells play and if adhesion may also occur in homophilic fashion, e.g. linkage of Tn to Tn EPs (cf. 136).

No quantitative studies on the problems of cell — cell recognition and adhesion of primary CA cells to their target (or substratum) seem to exist. The importance of temperature variation to dissect these initial events has been evaluated in other systems with varying results for different cell—cell interactions (91,92,134). We therefore must elucidate not only the overall event at physiological temperature in presence and absence of $Ca^{2+}$, a cation frequently important in adhesion (and, in preliminary experiments also in our systems), but we must also strive to quantitatively analyze reversible versus irreversible adhesion for which the proposed study of adhesion in temperature ranges from 4° to 37°C will be helpful as will be study over a range of different hydrogen ion concentrations. Dislodgement forces will be expressed in Dyne and determined as described by others (91,134).

(iii) Immunohistochemical Location of Tn and T EPs using Immunogold Techniques in Cell-Cell Adhesion.

We will use IHC to study the cell-cell systems described above both with LM as described below and with EM as described in section 2D.B.b1)(ii). Adhesion experiments will be performed in the absence of inhibitors and after addition of Tn and/or T in amounts which allow only partial adhesion to observe details of the adhesion process.

We will evaluate by IHC, Tn/T Ag distribution characteristics for appropriate cells adhering to various monolayers. Additional surgical specimens targeted for investigation consist of different types of malignant and benign cells: (a) Malignant, ductal, lobular and tubular CA cells, and among the ductal CAs well-differentiated cells versus anaplastic ones. (b) Among benign cells, hyperplastic, premalignant versus truly benign cells as defined by Dupont and Page (105).

The procedures outlined in Fig. 1 will be followed through completion of step D only. For IHC, the reaction systems will be sampled at appropriate times after initiation of cell-cell contact (i.e. before and after irreversible binding) e.g. at 30 min., 60 min., 90 min. Thereafter, the wells will be cut horizontally with a single edge razor and the segment of the wells containing the two monolayers will gently be inverted with a forceps and immersed in Petri dishes containing fixatives used for LM and EM. The cells will be fixed, rinsed, dehydrated and embedded while still within the well so as to retain the orientation of the cells.

Because the specimens of the adhesion layers are very small and the orientation is important, the resin JB-4 (Polysciences) is more suitable for embedment than paraffin. For LM, cells are fixed with Luftig's CB solution (123), washed with PBS, dehydrated in

Springer, Georg F., M.D.

Privileged Communication

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Application For Proposed Use of $^{51}Cr$ In The Investigation of Cell-Cell Adhesion

I. Proposed Use and Objectives:

Sodium [$^{51}Cr$] - chromate will be incorporated into cells of normal, benign, or malignant phenotype. The isotopically labelled cells will then be used to probe the qualitative and/or quantitative extent of their adherence to experimentally prepared monolayers of non-radiolabelled cells, both homotypic and heterotypic to the labelled cells.

II. Description of Procedure to be used:

1. The stock aqueous sodium [$^{51}Cr$] - chromate (1mCi/ml) container will be stored in a lead containment carrier. Personnel, wearing disposable gloves, will withdraw 50 uCi aliquots through the manufacturers serum stopper using a disposable tuberculin syringe.

2. $10^6$-$10^{10}$ cells will be collected by centrifugation in a disposable, plastic 15 ml conical centrifuge tube.

3. The cell pellet will be re-suspended in 0.5 ml of RPMI 1640, the $^{51}Cr$ aliquot added, and the mixture incubated 1 hr. at 37°C.

4. Following the incubation, the cells will be diluted with culture media (eg. RPMI 1640 + 10% FCS) and centrifuged. Following two additional washes in media, the final cell pellet will be suspended in 1 ml of media. Using a micropipettor, an aliquot will be withdrawn, the cells counted and tested for viability, and a dilution made dictated by that day's experiment.

5. Diluted cells will be distributed among several chambers of a cell-cell adhesion device. This device is constructed from two PVC microtiter plates joined face-to-face such that apposing wells form a continous chamber. Reagent additions are made through one end of the chamber and the chambers sealed prior to analysis. Labelled probe cells are brought into contact with the monolayer by centrifugation; subsequent to adhesion, the device is inverted and re-centrifuged. Finally, the device will be flash frozen and the chamber ends excised and counted.

III. Waste Handling and Disposal:

Gloves, pipets, pipet tips, culture tubes and titer plates will be sealed into a puncture-resistant container and disposed of as solid waste. Bulk liquids will be placed into an appropriate container as provided by the Office of Radiation Safety. All waste to be picked up by Radiation Safety personnel.

IV. Monitoring of Laboratory, Equipment and Personnel:

The experimentation area and centrifuge to be monitored weekly by smear wipe. Areas exhibiting radiation in excess of 200 DPM/100 $cm^2$ are subject to decontamination with subsequent wipes to fall below the 200 DPM/100 $cm^2$ limit. Personnel are to wear film badges to be submitted monthly for exposure densitometry.

V. Safety Analysis:

1. Chemical:
There are no inherent dangers. As supplied by the manufacturer, the aqueous solutions of sodium chromate are neither flammable nor explosive.

2. Biological:
The critical organ(s) for $^{51}Cr$ is total body wherein the biological half-life is 616 days. The maximum permissible body burden is 800 $\mu$Ci.

3. Radiological:
$^{51}Cr$ has a physical half-life of 27.7 days. The major source of emitted energy consists of a gamma particle of 0.32 MeV. During a single experiment the greatest exposure to gamma radiation would be on withdrawal of an aliquot from the stock container. At a distance of one foot, the exposure presented by a 1 mCi stock vial of $^{51}Cr$ would not exceed:

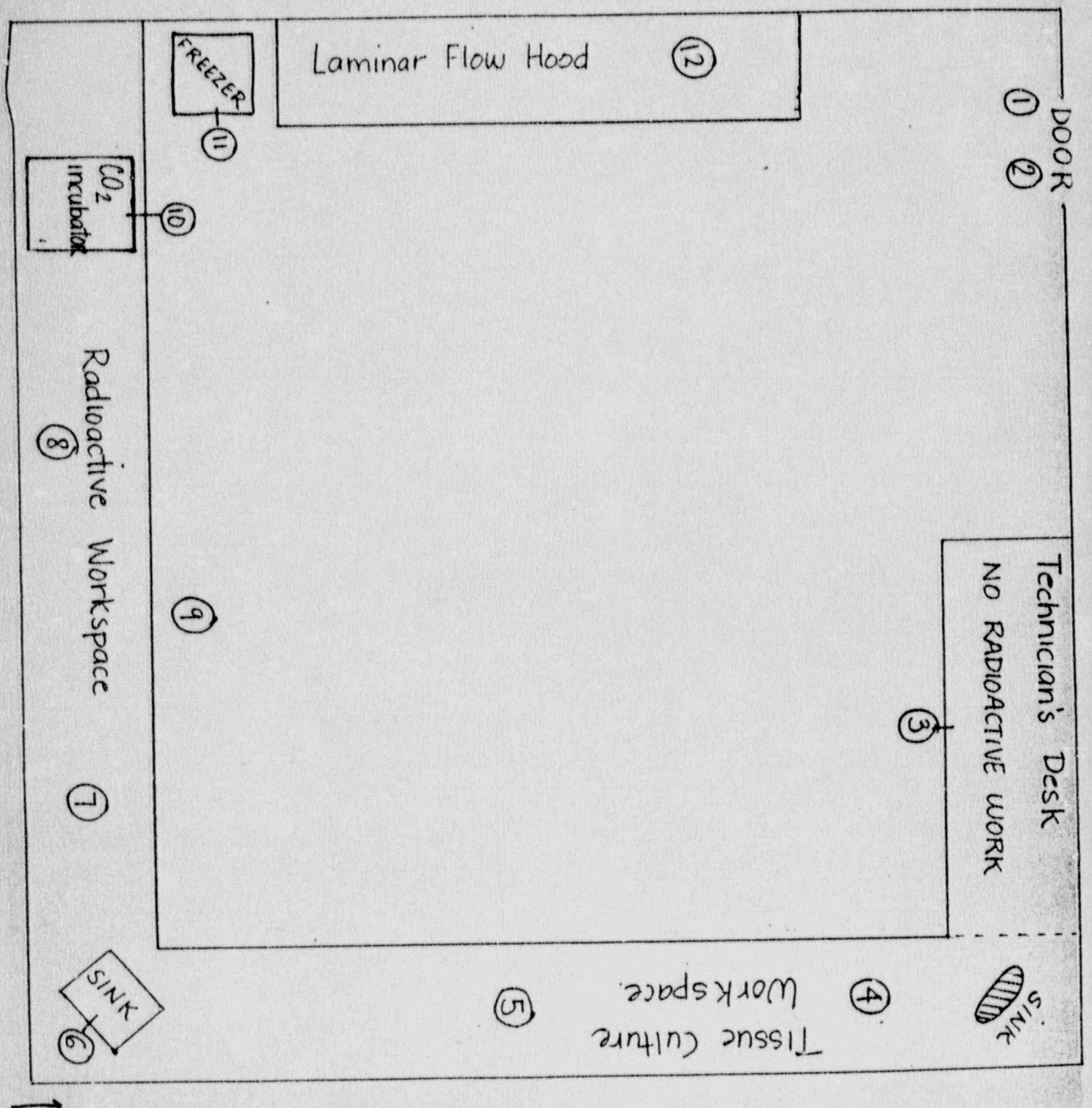
$$\frac{1.92 \text{ mR}}{\text{hr-mCi}} \times 1 \text{ mCi} = 1.92 \frac{\text{mR}}{\text{HR}}$$

The worst feasable accident would involve breakage of the stock container. In this event, contaminated personnel will be restricted to the immediate area for survey and decontamination. Non-essential personnel will be denied access. Office of Radiation Safety will be advised of the spill and their personnel will direct the proper disposal procedure.

Chicago Medical School/UHS

Microbiology/Immunology Department

V/A Bldg #1, Room 129 August, 1989




CONTROL NO. 87985

August, 1989

1. Smears of each numbered area will be done in duplicate.
2. Q-tips dipped in 95% EtOH will be used to wipe each indicated area.
3. Q-tips will be placed in scintillation vials, Gamma radiation will be detected by TM Analytic Gamma Counter.
4. Each surveyed area should be below 200 DPM/100cm$^2$
5. Any and all areas that exceed 200 DPM/100 cm$^2$ will be cleaned immediately, using appropriate decontamination techniques.
6. Results of the wipe tested areas will be made available to the Radiation Safety Office.

Areas to be tested:

① Floor of the doorway
② Handle of door
③ Technician's desk
④ T.C. Workspace
⑤ T.C. Workspace
⑥ Sink
⑦ Radioactive Workspace
⑧ Radioactive Workspace
⑨ Floor of Radioactive Space
⑩ $CO_2$ Incubator
⑪ Freezer
⑫ Laminar Flow Hood
⑬ Centrifuge in Rm 124
⑭ Gamma Counter in Rm 124

Karen M. Ridge

Prior Radioisotope Experience:

Professional Experience:

Chicago Medical School
Department of Biochemistry
North Chicago, IL
Lab Assistant II and Radiation Safety Officer of Owen's Lab.

Isotopes:

1. myo - [2 - $^{3}$H] Inositol CHOH $(CHOH)_3$ CHOH
Specific Activity 10-20 Ci/mmol, 370-740 GBq/mmol.

1uCi/ml $^{3}$H-inositol diluted in inositol-free EMEM media. To measure phosphoinositol release from vascular smooth muscle cells (VSMC).

Phosphoinositol (Pl) release from vascular smooth muscle cells (VSMC) monolayers was measured by trichloroacetic acid extracts that were placed on Dowex columns and eluted for Pl. 0.5 ml of each elutant and 3.5 ml of scintillation cocktail were counted on Packard 3200.

Culture medium and monolayer washes were disposed of as liquid waste. Tissue culture dishes, pipets, plastic columns were disposed of as dry waste. Scintillation fluid disposed as organic solvent.

Isotopes:

2. Rubidium-86-Specific Activity
1-8 mCi/mg Rb, 37-300 MBq/mg Rb

Isotope diluted in assay medium to 1uCi/mL. To measure the na/KlCl cotransport in vascular smooth muscle cells (VSMC) by quantitatively measuring $^{86}$Rb influx. VSMC incubated in $^{86}$Rb were extracted with 0.2% SDS. 0.5 ml extract and 3.5 ml scintillation cocktail were counted on Packard 3200.

University of
Health Sciences / The Chicago
Medical School

*Department of*
*Biological Chemistry*
*and Structure*

3333 Green Bay Road
North Chicago, Illinois 60064

Telephone
312.578.3221

November 16, 1988

Dr. Georg Springer
1800 Ridge
Evanston, IL 60201

Dear Dr. Springer:

I am writing to confirm that Karen M. Ridge has worked with radioisotopes while employed in my laboratory at the Chicago Medical School. Ms. Ridge has worked with $^{86}Rb$, $^{45}Ca$, and $^{3}H$. She has also been responsible for management of all radioactive waste and for routine laboratory monitoring. I have carefully trained her in all these procedures. I am confident of her ability to safely and responsibly carry out experiments using radioisotopes.

Sincerely yours,

Nancy E. Owen

Nancy E. Owen, Ph.D.
Associate Professor

NEO/jw

Culture medium disposed as liquid waste. Tissue culture dishes, pipet tips disposed as dry waste. Scintillation fluid disposed as organic solvent waste.

3. Calcium 45 Specific Activity 10-40 mCi/mg Ca, 0.37-1.5 GBq/mgCA.

Isotope diluted in assay medium to 1uCi/mL to measure the influx of extracellular calcium and calcium as a second messenger in VSMC. The experimental protocol involving Ca-45 paralleled that of the Rb-86 experiments above.

March, 1989

# GEORG F. SPRINGER, M.D.

## CURRICULUM VITAE

Born February 29, 1924, Berlin, Germany, oldest son of medical-scientific publisher Dr. h.c. Ferdinand Springer and Elisabeth, née Kalvin (Springer-Verlag Publishing Co., founded 1842). 1942-45, German Army, 1942-43, Russia, tank driver. Wounded twice, once seriously. U.S.-P.O.W. 1945. 1941 & 1949, saved two people from drowning. 1961, U.S. citizen.

### EDUCATION AND PROFESSIONAL EXPERIENCE

1930-34, Public School, Berlin. 1934-42, High School: Kaiserin-Auguste Gymnasium (humanistic).

1945-48, U. of Heidelberg, Germany. 1946, president of student committee representing U. of Heidelberg students before U.S. Military Government and University. 1946-47, founding and opening of Heidelberg branch of Springer-Verlag Co. 1947, M.S, Medicine, summa cum laude.

1948-51, U. of Basel, Switzerland. - M.D. summa cum laude. Extracurricular training in tumor histology. 1949-51, thesis under Prof. E. Freudenberg and extracurricular training in organic chemistry under Prof. T. Reichstein (Nobel Laureate). 1951, Internship.

1951-62, U. of Pennsylvania, Philadelphia. 1951-54, Research Fellow & Resident in Pathology. 1954-56, Immunochemist, joint germfree research project Army Medical Service Graduate School (Walter Reed), Washington, D.C. and U. of Penn. 1956-61, Assist. Prof. Immunopathology. 1961-62, Assoc. Prof. Pathol. & Microbiol., U. of Penn. Research Immunologist in charge of Blood Banks and Serology, U. of Penn. Hospital. Advisor, Clinical Research Center, Phila. General Hospital. 1961-62, Chief Blood Bank Examiner, City of Philadelphia.

1963-69, Prof. Microbiology-Immunology, Northwestern U. Medical & Dental Schools, Chicago. Director, Dept. Immunochemistry Research, Evanston Hospital, Evanston, IL.

1977-89, Prof. Surgery & Julia S. Michels Investigator in Surgical Oncology.

### AWARDS

| | |
|---|---|
| 1966 | Oehlecker Prize, German Soc. Blood Transfusion (for clinical and immunochemical discoveries in blood group field). |
| 197 | Abbott Laboratories Award for Fundamental Contributions - Biomedicine (for fundamental contributions to immunology of breast cancer) |
| 1977 | Ernst Jung Prize for Medicine (for immunologic detection of breast carcinoma). Highest German award in biomedical sciences. |
| (1986 | Proposed for Bristol-Myers Award) not obtained. |

### FELLOWSHIPS

| | |
|---|---|
| 1952-53 | Woodward Fellow, Biochemistry |
| 1958-63 | Established Investigator, American Heart Association |

### EDITORSHIPS (Primary or advisory)

a) Journal of Tumor Marker Oncology
b) CRC Critical Reviews in Oncogenesis
c) Pathology and Immunopathology Research
d) Immunologic Research
e) Medical Chemistry Progress
f) Protoplasmatologia
g) Progress in Molecular and Subcellular Biology
h) Progress in Clinical Biochemistry and Medicine

Over 380 publications.

(more)

MEMBERSHIPS IN SOCIETIES

| | |
|---|---|
| Fellow: | Am. Assoc. Advancement Science; Am. Inst. Chemists |
| Honorary Member: | Illinois State Academy of Science |
| Member: | Am. Assoc. Cancer Research; Am. Assoc. Immunologists; Am. Chemical Soc.; Am. Fed. for Clinical Research (senior member); Am. Soc. Biological Chemists; Advisory Board, Am. Soc. Clinical Microbiology; British Biochemical Soc.; French Immunological Soc. (life member); German Soc. Immunology; Scientific Advisory Council, Intl. Academy Tumor Marker Oncology; Intl. Assoc. Breast Cancer Research; Intl. Soc. Differentiation; Intl. Soc. Glycoconjugates; Northwestern Univ. Cancer Center (founding member); The Protein Society; Chicago Thoracic Society; The Gerontological Society of America. U.S. Equestrian Team, 1970-79; Nature Conservancy (Natl. and Intl., life member); Natl. and Intl. Wildlife Federations; Chicago Council on Foreign Relations. |
| Consultant: | Aug., 1988-Advisor, German Cancer Research Center, Heidelberg, W. Germany; U.S. Natl. Cancer Inst. Organ Systems Working Group: Cellular Adhesion (1986-88); The Center on Aging, Northwestern Univ. Medical School. |

At present, and at their initiative, the following collaborate with us:

1) Biomembrane Institute & Wash. Univ., Seattle, Washington
2) Cambridge Univ., England
3) Creighton Univ., Omaha, Nebraska
4) Danish Natl. Cancer Center
5) Institute of Breast Diseases, Westchester County Med. Center, Valhalla, New York
6) Medical Biology Institute, La Jolla, California
7) Montreal General Hospital
8) Univ. of Southern California
9) Weizmann Institute of Sciences, Israel

IMPORTANT SEMINARS AND MEETINGS FROM 1985 TO 1989

1985 By invitation of President of Weizmann Institute, Rehovot, Israel, two seminars; Scripps Institute, La Jolla, CA; Duke University, Durham, NC.

1986: National Cancer Institute's Breast Cancer Working Group, Bethesda, MD, Chairman of proposal meeting; Plenary lecture at 3rd Intl. Conf. on Human Tumor Markers, Ischia, Italy; German Cancer Research Center, Heidelberg, W. Germany; University of Düsseldorf, W. Germany; University of Cambridge, England; Oncogen, Div. of Bristol-Myers, Seattle, WA.

1987: The Biomembrane Institute, Seattle, WA; Jung Foundation for Science and Biomedical Research, Hamburg, W. Germany; 4th Intl. Conf. on Human Tumor Markers, New York, NY; Bat-Sheva Seminar on Pathogenesis and Prevention of Tumor Dissemination, Rehovot-Eilat, Israel (organizer: Weizmann Institute).

1988: Advisor, German Cancer Research Center, Heidelberg, W. Germany, with special immediate task to participate in the Restructuring of the Cancer Center's Institute for Toxicology and Chemotherapy.

1989: Blood Group and Other Carbohydrate Antigens in Human Epithelial Cancer Workshop, Memorial Sloan-Kettering Cancer Institute: Pan carcinoma T and Tn autoantigens are adhesion molecules and functional predictors of tumor aggressiveness; 5th Annual Meeting of SEK, German Cancer Society, Heidelberg, W. Germany: Pan carcinoma T and Tn epitopes are diagnostic molecular markers and autoimmunogens that detect incipient carcinomas and discriminate between patients with reasonable and with poor prognosis; 7th Intl. Congress of Immunology, Berlin, W. Germany: T & Tn pan carcinoma autoantigens: Fundamental and clinical aspects.

FAMILY

Two brothers, one sister. Married, 1951, Heather Margaret Bligh of Caterham, Surrey, England (deceased, 1980: breast carcinoma). Children: Martin F.B., M.D., Emergency Medicine, Mt. Sinai Hospital; Elizabeth A., M.D., Senior Resident, Northwestern U.; Julia A., Law student, U. of Virginia, Charlottesville, VA.

August 29, 1989

Chairman, Radiation Safety Committee (115)

Research Proposal

Georg F. Springer, M.D.

1. This is to inform you that your project entitled, "Carcinoma Pathogenesis: Role of T and Tn Antigens" was approved by the Radiation Safety Committee.

2. From the aspect of radiation safety, you may begin your project.

GREGORY A. GERGANS, M.D.

cc:
Medical Research Service (151)
Radiation Safety Officer (11B)

CONTROL NO. 87985