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DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

APPLICATION
FOR CONTINUATION GRANT

REVIEW GROUP		PROGRAM	GRANT
CAP	5	P01	CA-14052-02

TOTAL PROJECT PERIOD
From: 06/30/73 Through: 05/31/76

REQUESTED BUDGET PERIOD
From: 06/01/74 Through: 05/31/75

1. TITLE
Preclinical Studies for Pion Radiotherapy

2A. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Name and Address, Street, City, State, Zip Code)
Kligerman, Morton M.
Cancer Research and Treatment Center
University of New Mexico
Albuquerque, New Mexico 87131

4. APPLICANT ORGANIZATION (Name and Address-Street, City, State, Zip Code)
Cancer Research and Treatment Center
University of New Mexico
Albuquerque, New Mexico 87131

2B. DEGREE
M.D.

2C. SOCIAL SECURITY NO.
[redacted]

5. PHS ACCOUNT NUMBER
726225

2D. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Cancer Research and Treatment Center

2E. MAJOR SUBDIVISION
Division of Health Sciences

3. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES
20 Cancer Research and Treatment Center

6. TITLE AND ADDRESS OF OFFICIAL IN BUSINESS OFFICE OF APPLICANT ORGANIZATION
Carroll J. Lee, Comptroller
University of New Mexico
Albuquerque, New Mexico 87131

7. RESEARCH INVOLVING HUMAN SUBJECTS (See Instructions)
☒ No ☐ Yes APPROVED: _____ Date

8. INVENTIONS (See Instructions)
☒ No ☐ Yes-not previously reported
☐ Yes-previously reported

9. PERFORMANCE SITE(S)
Cancer Research and Treatment Center
University of New Mexico
Albuquerque, New Mexico 87131
Congressional District #1

Los Alamos Scientific Laboratory
Los Alamos, New Mexico 87544
Congressional District #1

10. DIRECT COSTS REQUESTED FOR BUDGET PERIOD
$754,380

TELEPHONE INFORMATION

	Area code	Tele No & Ext
11A. PRINCIPAL INVESTIGATOR Kligerman, M.M. OR PROGRAM DIRECTOR (Item 2a)	505	277-3631
11B. Name of business official (Item 6) Carroll J. Lee Comptroller	505	277-5111
11C. Name and title of administrative official (Item 15b) Carroll J. Lee Comptroller	505	277-5111

12A. CONGRESSIONAL DISTRICT OF APPLICANT ORGANIZATION SHOWN IN ITEM 4
Congressional District #1

12B. COUNTY OF APPLICANT ORGANIZATION SHOWN IN ITEM 4
Bernalillo County

13. DO NOT USE THIS SPACE

REPOSITORY LANL/ARC
COLLECTION MP-DO
BOX No. A-91-011
FOLDER 179-9

14. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

15. SIGNATURES
(Signatures required on original copy only. Use ink. "Per" signatures not acceptable.)

15A. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR — DATE

15B. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION — DATE

PHS 2590-1 (All previous editions obsolete)
REV 1-70

FILE BARCODE
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SECTION -BUDGET (0 Y 12 MONTHS) 06/01/74 /31/75 CA-14052-02

A. ITEMIZE DIRECT COSTS REQUESTED FOR NEXT BUDGET PERIOD

PERSONNEL NAME (Last, First, Initial) (a)	TITLE OF POSITION (b)	TIME OR EFFORT %/HRS. (c)	SALARY REQUESTED (d)	FRINGE BENEFITS (See Instructions) (e)	TOTAL (f)
Kligerman, M.M., M.D.	PRINCIPAL INVESTIGATOR	40	None (LASL-UNM Support)		
Barnes, J.E., Ph.D.	Radiation Physicist	40	8,440	1,265	
New Hire	Radiation Physicist	60	10,550	1,575	
Key, C.R., M.D.	Histopathologist	10	None (UNM Support)		
Jordan, S.W., M.D.	Histopathologist	10	None (UNM Support)		
New Hire	Histopathologist	60	15,000	2,250	
Palmer, Susan	EM/Histotechnician	100	6,930	1,040	
Wilson, S.	Grant Administrator	34	5,245	785	
Guana, L.	Secretary	40	2,400	360	
	Subtotals →		$48,565	$7,275	

(Indicate cost of each item listed below) TOTAL (Columns (d) and (e)) → $ 55,840

CONSULTANT COSTS (See instructions)

Visiting Scientists (see attached) 50,000

EQUIPMENT

Physics and histopathology equipment 5,000

SUPPLIES

Animals (purchase and care) $2,000
Electron microscopy and histo-pathology supplies 1,000

$ 3,000

TRAVEL

DOMESTIC Travel to Los Alamos $ 500

FOREIGN None

PATIENT COSTS (See instructions)

None

ALTERATIONS AND RENOVATIONS

None

OTHER EXPENSES (Itemize)

See attached (work to be performed by Los Alamos Scientific Laboratory) 640,040

TOTAL DIRECT COST (Enter on Page 1, Item 10) $ 754,380

INDIRECT COST (See instructions)

_____% S&W*
_____% TDC*
*If this is a special rate (e.g. off-site), explain.

Date of DHEW Agreement: _____

☐ Not Requested
☐ Under negotiation with

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CONSULTANT COSTS:

Consultant	Specialty Field	Percent of Time
M. Boone, M.D.	Radiotherapy	15
W. Powers, M.D.	Radiotherapy	15
J. Concannon, M.D.	Radiotherapy	10
S. Levitt, M.D.	Radiotherapy	10
K. Loeffler, M.D.	Radiotherapy	10
R. Stewart, M.D.	Radiotherapy	10
C. von Essen, M.D.	Radiotherapy	10
E. Gillette, D.V.M.	Animal Radiobiology	15
A. Wiley, M.D.	Tumor Radiobiology	10
R. Withers, M.D.	Cellular Radiobiology	15
B. Bjarngard, Ph.D.	Radiological Physics	5
W. Hendee, Ph.D.	Radiological Physics	5
A. Koehler, Ph.D.	Radiological Physics	5
L. Lanzl, Ph.D.	Radiological Physics	5
J. Laughlin, Ph.D.	Radiological Physics	5
R. Loevinger, Ph.D.	Radiological Physics	5
R. Shalek, Ph.D.	Radiological Physics	5

OTHER EXPENSES*:

Contract with Los Alamos Scientific Laboratory, for the following:

1. Personnel Costs (salary and fringe):		$318,160
Health Research Division	$213,000	
Medium Energy Physics Division	105,160	
2. Equipment:		75,200
Health Research Division	$ 25,800	
Medium Energy Physics Division	49,400	
3. Supplies:		53,300
Health Research Division	$ 38,300	
Medium Energy Physics Division	15,000	
4. Travel		8,800
5. Other (shops services, computer services, etc.)		22,000
6. Indirect costs (at 51.1% of personnel costs)		162,580
TOTAL		$640,040

*Detailed breakdown for Los Alamos Scientific Laboratory contained on following budget pages.

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A. ITEMIZE DIRECT COSTS REQUESTED FOR THAT BUDGET PERIOD LOS ALAMOS SCIENTIFIC LABORATORY BUDGET

PERSONNEL NAME (Last, First, Initial) (a)	TITLE OF POSITION (b)	TIME OR EFFORT %/HRS. (c)	SALARY REQUESTED (d)	FRINGE BENEFITS (See instructions) (e)	TOTAL
Kligerman, M.M., M.D.	PRINCIPAL INVESTIGATOR	40	None (UNM-LASL Support)		
Rosen, L., Ph.D.	Deputy Principal Investigator	10	None (LASL Support)		
Voelz, G.L., M.D.	Deputy Principal Investigator	15	None (LASL Support)		
LASL Health Research Division (see attached)					
LASL Medium Energy Physics Division (see attached)					
	Subtotals →		$	$	

(Indicate cost of each item listed below) TOTAL (Columns (d) and (e)) → 318,160

CONSULTANT COSTS (See instructions)

None

EQUIPMENT

See attached 75,200

SUPPLIES

See attached 53,300

TRAVEL

DOMESTIC 8,800

FOREIGN None

PATIENT COSTS (See instructions)

None

ALTERATIONS AND RENOVATIONS

None

OTHER EXPENSES (Itemize)

Shops services, computer services, etc.	$ 22,000
Administrative costs (indirect costs) at 51.1% of personnel costs	162,580
	184,580

TOTAL DIRECT COST (Enter on Page 1, Item 18) 640,040

INDIRECT COST (See instructions)

_____% S&W*

_____% TDC*

Date of DHEW Agreement: _____

☐ Not Requested

☐ Under negotiation with

*If this is a special rate (e.g. off-site), explain. _____

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LOS ALAMOS SCIENTIFIC LABORATORY BUDGET: CA-14052-02

PERSONNEL COSTS:

Name	Title of Position	% Time	Salary Requested	Fringe Benefits	Total
Health Research Division:					
					$213,000
Medium Energy Physics Division:					
					105,160
			TOTAL PERSONNEL COSTS		$318,160

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LOS ALAMOS SCIENTIFIC LABORATORY BUDGET (CONTINUED): CA-14052-02

EQUIPMENT COSTS:

Health Research Division:

Operating and necropsy table	$ 800
Portable surgical light	300
Mayo instrument stand	100
Intravenous stand	100
Animal isolation and transport cages	1,800
Inverted microscope	1,000
CO_2 Incubators (2)	2,500
Standard Zeiss Microscope	2,500
Lens for Zeiss Microscope	1,700
Magnetic tape compatible with Canberra analyzer	10,000
Interface for magnetic tape	3,000
Amplifiers and assorted equipment	2,000
Subtotal	$25,800

Medium Energy Physics Division:

Oscilloscopes (2)	6,500
CAMAC interface modules	6,000
Electrostatic printer plotter	6,000
Proportional counter	4,000
Ionization chambers	4,000
Solid state detectors	2,000
Flow system	4,000
Vacuum pumps	2,500
Vacuum gauges and controls	1,500
Fast nuclear electronics	4,900
Slow nuclear electronics	8,000
Subtotal	$49,400
TOTAL EQUIPMENT COSTS	$75,200

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LOS ALAMOS SCIENTIFIC LABORATORY BUDGET (CONTINUED):

SUPPLY COSTS:

Health Research Division:

Animals	$ 4,800
Animal colony expendables	3,500
Animal food and fodder	2,500
Animal cages	5,000
Histopathology supplies	2,500
Clerical and Xerox supplies	2,500
Shop materials, exposure casettes, etc.	3,000
Cell culture media	4,000
Labeled compounds	1,000
Chemicals	2,000
Glassware and plastic ware	5,000
General laboratory supplies	2,500
Subtotal	$38,300

Medium Energy Physics Division:

Prototype logarithmic	3,000
Tissue equivalent plastics	5,000
Cables	500
Computer system maintenance	5,000
Lab supplies, wire, tools	1,500
Subtotal	$15,000
TOTAL SUPPLY COSTS	$53,300

8. Supplemental information regarding ITEMS in the proposed budget for the next period which require explanation or justification. (See instructions)

Explanation of the purpose of the various budget items was included in the original grant request. It should be noted that the total amounts for equipment and supplies (for UNM and LASL) differ slightly from the amounts recommended for Year 2 by the NCI Review Council. This is a result of realignment of the two lists to ensure that no capital equipment items are listed with supply items.

Most of the equipment items have been recommended for purchase at LASL, since they will be in use at LASL, to shorten the time required between initial request and final delivery. Ownership of capital equipment items will be retained by UNM, however, as defined by NCI and the contract between UNM and the University of California for operation of this grant.

Because of the increased workload anticipated during Year 2 with actual use of the pion beam, UNM is requesting addition of one radiation physicist (60 percent) to ensure that the equivalent of one physicist will be working full time at the Biomedical Facility in Los Alamos. Additional histopathology support (60 percent of a new pathologist's time) is requested for the same reason.

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SECTION I. FISCAL DATA FOR CURRENT BUDGET PERIOD (USUALLY 12 MONTHS)	FROM 06/30/73	THROUGH 05/31/74	CA-14052-02

The following pertains to your CURRENT PHS budget. Do not include cost sharing funds. This information in conjunction with that provided on Page 2 will be used in determining the amount of support for the NEXT budget period.

A. BUDGET CATEGORIES		CURRENT BUDGET (As approved by awarding unit) (1)	ACTUAL EXPENDITURES THRU 12/31/73 (Insert Date) (2)	ESTIMATED ADDITIONAL EXPENDITURES AND OBLIGATIONS FOR REMAINDER OF CURRENT BUDGET PERIOD (3)	TOTAL ESTIMATED EXPENDITURES AND OBLIGATIONS (Col. 2 plus Col. 3) (4)	ESTIMATED UNOBLIGATED BALANCE (Subtract Col. 4 from Col. 1) 5
Personnel (Salaries)		27,604	11,382	13,450	27,604	0
Fringe Benefits		4,141	1,244	2,018	4,141	0
Consultant Costs		22,500	732	21,768	22,500	0
Equipment		16,575	2,781	13,794	16,575	0
Supplies		5,000	3,604	1,396	5,000	0
TRAVEL	Domestic	2,500	1,101	1,399	2,500	0
	Foreign					
Patient Costs						
Alterations and Renovations						
Other		523,825*	153,290	370,535	523,825	0
Total Direct Costs		602,145	174,134	428,011	602,145	0
Indirect Costs (If included in award)						
TOTALS →		$602,145	$174,134	$428,011	$602,145	$ 0

Use space below to:

B. List all items of equipment purchased or expected to be purchased during this budget period which have a unit cost of $1000 or more.

C. Explain any significant balance or deficit shown in any category of Column 5.

D. List all other research support for Principal Investigator by source, project title, and annual amount.

* Per negotiation, these funds specified as follows:

Personnel	$221,900
Supplies	73,000
Travel	10,000
Other	46,500
Adm. Services (Indirect costs)	138,800

In addition, $33,625 was transferred from EQUIPMENT (UNM) to OTHER (LASL) for equipment purchase at LASL (with NCI approval). Also, $2,000 of LASL's travel funds designated for foreign travel with NCI approval.

B. Items of Equipment Purchased Which Have a Unit Cost of $1,000 or More:

Isodose-isodensity plotter system	$16,500
Printer plotter for treatment plan output	10,682
Computer storage display scope and keyboard	3,868

(See attached)

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B. Items of Equipment Purchased Which Have a Unit Cost of $1,000 or More (Continued):

Digitizer table	$2,575
Closed circuit television equipment	2,781
Total	$36,406

Items of Equipment Expected to Be Purchased Which Have a Unit Cost of $1,000 or More:

Amplifier and Preamps	$ 2,800
A to D Connectors	1,900
X-ray film processor	7,000
Densitometer	1,500
Total	$13,200

C. None

D. Other Research Support for Principal Investigator:

NCI 1-P01-CA-14734-01: Cancer Center Program, $787,390 (current year), $1,961,922 (total for entire project), approved but not yet funded.

NCI 5-P01-CA-11998-02: Regional Plan for Meson Radiotherapy of Cancer (by letter request $9,500 budgeted for extension of grant period through 31 March 1974).

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SECTION IV—SUMMARY PROGRESS REPORT	CA-14052-02	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
Kligerman, Morton M.	FROM	THROUGH
NAME OF ORGANIZATION	06/30/73	01/15/74
Cancer Research & Treatment Center, Univ. of N.M.		
TITLE (Repeat title shown in Item 1 on first page)		
Preclinical Studies for Pion Radiotherapy		

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

1. PUBLICATIONS

(a) Published: ~~None~~

(b) In Press:

Kligerman, M.M., and Petersen, D.F. Biomedical Program Leading to Therapeutic Trials. Proceedings of the Joint Meeting of the Radiological Society of North America and American Association of Physicists in Medicine, Chicago, Illinois, November 25-30, 1973 (copy attached).

Knapp, E.A. Meson Factories, Pi Meson Delivery, and Pi Meson Dosimetry for Cancer Therapy. Proceedings of the Joint Meeting of the Radiological Society of North America and American Association of Physicists in Medicine, Chicago, Illinois, November 25-30, 1973 (copy attached).

Raju, M.R. Negative Pions in Radiotherapy: A Brief Review. Eur. J. Cancer (Talk presented at the Second Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy, The Hague, The Netherlands, October 3-5, 1973) (copy attached

Raju, M.R. The Biological Effects of Negative Pions. Proceedings of the XIIIth International Congress of Radiology, Madrid, Spain, October 15-30, 1973 (copy attached).

Shlaer, W.J. PIPLAN: A Radiation Therapy Treatment-Planning Code for Pions. Los Alamos Scientific Laboratory Report, Los Alamos, New Mexico, January 1974.

(c) Abstracts:

Kligerman, M.M., and Petersen, D.F. Biomedical Program Leading to Therapeutic Trials. RSNA Scientific Program 1973, 59th Scientific Assembly and Annual Meeting, Radiological Society of North America and American Association of Physicists in Medicine, Chicago, Illinois, November 25-30, 1973, Abstract No. 86, p. 124.

Kligerman, M.M., and Petersen, D.F. Program Leading to Clinical Trials at LAMPF. Abstract for Discussion Session VI, Plans for Neutrons and Negative Pi Meson Radiotherapy at Various Centres, Second Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy, The Hague, The Netherlands, October 3-5, 1973.

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Knapp, E.A. Possible Pion Sources for Radiotherapy. Abstract for Discussion Session VI, Plans for Neutrons and Negative Pi Meson Radiotherapy at Various Centres, Second Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy, The Hague, The Netherlands, October 3-5, 1973.

Raju, M.R. Biological Effects of Negative Pions. Excerpta Medica, International Congress Series No. 301, XIIIth International Congress of Radiology, Symposium on the Present Status of Therapy with Pi Mesons, Madrid, Spain, October 15-20, 1973, Abstract No. 291, p. 97.

2. PERSONNEL CHANGES

(a) University of New Mexico:

Scott Jordan, M.D., Associate Professor of Pathology, substituted for R. E. Anderson, M.D., Associate Professor and Chairman of Pathology. (Biographical sketch for Dr. Jordan attached.)

(b) Los Alamos Scientific Laboratory:

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SUMMARY PROGRESS REPORT

1. Objectives

The primary objective of this project is to provide, as soon as possible, information crucial to the radiotherapist for beginning clinical trials of pion radiation therapy for patients with advanced cancer. Specific objectives are:

a. To develop, test, and apply methods of pion dosimetry that are essential to the radiobiology experiments and which will logically carry over into the early pilot trials and subsequent clinical trials of the efficacy of pion therapy for cancer patients.

b. To employ appropriate quantitative cellular radiobiology techniques to establish the relative biological effectiveness (RBE), oxygen enhancement ratio (OER) and effects of dose fractionation of pions under radiation conditions and geometries most informative to the radiotherapist, and

c. To observe in appropriate experimental animals early and late responses and radiopathology of normal and tumor tissues exposed to various segments of the pion beam path to assess acute effects and delayed sequelae as a function of dose and exposure.

Individual goals for each of the three project years were not established, as achievement of the objectives listed above must progress in an orderly manner, contingent upon other factors in the development of the biomedical facility at the Los Alamos Meson Physics Facility (e.g., completion of the biomedical facility and activation of the pion biomedical beam). Pions were first obtained at Los Alamos on 26 August, 1973, and activation of the biomedical channel is expected on 26 January 1974. Although initiation of the radiobiology studies with the pion beam awaits activation of the biomedical channel, considerable headway has been made in establishing baseline data for cell systems and animal tissue (both normal and tumorous), using 250 KVP x-rays as controls. Priorities for continuing experiments have been established according to the intensity levels of the beam expected to be reached during sebsequent phases of development.

2. Current Studies

This section describes studies conducted at Los Alamos Scientific Laboratory (LASL) and the University of New Mexico Cancer Research and Treatment Center since the start of the grant period on 30 June 1973 to approximately 15 January 1974.

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a. Biomedical Beam Operation

The magnetic channel that collects, purifies, and directs on the patient the beam of negative pions for therapy is now complete enough to start the tuning with a pion beam, and all components of the channel should be installed during the next shutdown of the LAMPF accelerator which is scheduled for March 1974. The tuning apparatus is now constructed and is being checked out in preparation to tuning the channel in the next few weeks.

All computer control system hardware has been installed and computer executive and operating programs are currently being tested. Dosimetry equipment required to begin initial biological experiments on the pion beam is under development. A comprehensive treatment planning program has been developed which will allow the depth-dose distribution to be calculated for various pion beam arrangements in combinations of tissue, bone, air space, and other density inhomogeneities in the patient. Output from the code is in the form of two-dimensional isodose maps for planes at any angle through the treatment volume.

b. Cellular and Mammalian Radiobiology

(1) Spatial Distribution of Mammalian Cell Lethality. Using a gelatin method of suspension, cultured mammalian cells in three-dimensional configurations have been irradiated either end-on or side-on with 250 KVP x-rays. In all test cases, cell survival (based on subsequent colony formation) followed that predicted from the x-ray beam profile and depth-dose profile. It is clear that this will be one of the methods of choice in pion irradiations of cultured cells. Other methods of cell holding that have been investigated include culture in agar and allowing colonies to develop in situ before the gel is sliced for scoring. A study has also been made of our ability to hold cells at lower than normal temperatures for extended time periods--a condition anticipated in early pion irradiations. Survival experiments employing these conditions will probably be the earliest biological experiments performed with the low-intensity pion beam.

(2) Effect of Mixed Radiations on Cell Survival in the Presence and Absence of Oxygen. Cultured mammalian cells have been irradiated with various mixtures of alpha particles from plutonium-239 and 250-KVP x-rays. Results to date indicate that small admixtures of alpha particles lead to small enhancements of cell killing but to very significant reductions in oxygen enhancement ratio (OER). It is of particular interest to understand the effects of different radiation mixtures, as the portion of stopping pions will be inverseley related to the treatment volume.

(3) Cell-Cycle Dependence of Survival at High Linear Energy Transfer. Chinese hamster cells were synchronized by mitotic selection and irradiated with 250-KVP x-rays or plutonium alpha particles at hourly intervals throughout the cell cycle. At two different doses, colony-formation survival was independent of cycle stage following alpha particle irradiation and was strongly

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dependent upon cycle stage following x-irradiation. Progress is being made in obtaining synchronous cultures of human kidney cells so that similar studies can be conducted with human tissue.

(4) Early and Late Effects in the Acutely Irradiated Mouse Foot. Erythema and desquamation of the skin of the mouse paw and loss of nails and toes, as used by S.B. Field at Hammersmith in London, have been adopted for early use in pion studies, since rapid results can be obtained. A prelimary set of experiments has been performed in which paws of mice were irradiated with 250-KVP x-rays and graded for erythema and desquamation by a traditional graded scale. A dose-effect curve that satisfactorily resembled Field's results for single exposures was obtained, for both acute and late effects. Studies using fractionated exposures will also be initiated, as these early skin experiments may be the most useful measurements for establishing early patient exposure conditions.

(5) KHT Tumor Regression with Fractionated Doses of X-Rays. A series of single-dose experiments has already been completed in which skin reactions and tumor regrowth were evaluated in KHT sarcoma-bearing mice. Irradiations have also been delivered (250-KVP x-rays) at three fractions per week, and it was found that the tumor doubling time following a given total dose was shorter, as expected, when the dose was fractionated. A mouse holder has been designed and tested for continuing these experiments with the pion beam.

(6) Development of the EMT Mouse Tumor System for Radiobiology. The EMT mouse tumor cell line has been chosen for our studies with pions, as it will also be used in similar studies at Stanford. It is possible to irradiate these cells *in vivo* or *in vitro* under hypoxic or oxygenated conditions and to assay them for viability *in vivo* or *in vitro* using tumor formation (100 days) or colony formation (10 days) as the respective end points. The tumor cell line has now been growing successfully *in vivo* and *in vitro* for about one year, and an experiment has been performed in which EMT tumor cells were irradiated in culture, in living mice, and in recently killed mice, with this last condition defining the anoxic state. Colony-formation survival curves were obtained and were typical of fully oxygenated, mixed, and fully anoxic populations, respectively. In preparation for more extensive studies with pions, x-ray experiments with this cell line are continuing.

(7) Radiobiology of Vicia faba Root Meristems. The radiation sensitivity of the root growth rate in seedlings of the broad bean *Vicia faba* makes it ideal for studies of radiation effects at moderate and low doses, and its reputation for reliably measuring OER in neutron beams has been establihed. Holders have been built, and x-ray exposures in the presence and absence of oxygen have been performed, using both the traditional British strain and locally grown strains of *Vicia*, and the anticipated results were obtained. This system is ready for pion experiments at any time.

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(8) Preliminary Studies for Pion Biomedical Program. Studies under way include work with three transplantable mouse tumors, skin studies using two models, and ancillary work with jig development. An experiment to establish the tumor control dose-50 for the C3H mammary carcinoma has been started. The tumor is being studied using 250-KVP x-rays under hypoxic, ambient, and hyperbaric conditions using 60 animals per group. A second study with a fractionated exposure scheme is in its initial stages. Preliminary skin experiments with 250-KVP x-rays have begun, using pigs as subjects. Dose effects can be studied with up to six dose patches per animal. Several prototype animal holding jigs have been developed which offer the capability of exposing various numbers and sizes of animals. Blood gas studies have been performed on mice to determine the effects of anesthesia and confinement on blood oxygen and carbon dioxide levels.

(9) Ultrastructural Pathology of Irradiated Kidney--Late Effects. Three questions are being addressed with respect to the morphologic changes following renal irradiation: (a) What light and electron microscopic changes occur and at what time intervals after irradiation? (b) What is the RBE of pion vs. neutron vs. conventional irradiation causing a given degree of pathologic alteration? (c) Can terminal structure and/or function of kidney be predicted relatively quickly after irradiation from its morphologic appearance? Mice studies, using varying doses of 250 KV x-rays up to 2250 rads, were begun in 1972, and light and electron microscopic evaluation of renal histologic changes are currently being carried out on tissue from animals sacrificed at specified intervals after irradiation. The experiments will be repeated using higher doses of 250 KV irradiation because of the paucity of changes seen to date, and using pion and perhaps neutron evaluation as these modalities become available.

3. Significance

This project is intended to develop the technology to establish whether negative pions are indeed as promising for cancer therapy as present knowledge suggests, in terms of such factors as depth-dose distribution, low-LET in intervening normal tissue, finite range, and sophisticated exposure control. These investigations are an essential prerequisite to pilot studies and subsequent controlled clinical trials of pion radiotherapy for cancer patients.

4. Research Goals for Year 2

Research goals for Year 2 are summarized as follows:

a. Biomedical Beam Operation

(1) Refinements in tuning of the pion beam.

(2) Continued improvement of the computerized treatment planning program and modification of the program to allow it to operate on the PDP 11/45 computer at the biomedical facility.

(3) Initiation of dosimetry studies, using the pion biomedical channel.

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b. Cellular and Mammalian Radiobiology. Priorities outlined below reflect both the importance of the information to be received and the practical aspects of efficiently utilizing a beam that will increase in intensity from a few rads per minute to full intensity over a period of 12 to 24 months.

(1) Cellular Radiobiology

(a) Initial RBE studies to establish reliable RBE values as a function of depth in tissue-equivalent material.

(b) Studies to obtain quantitative estimates of the oxygen enhancement ratio (OER) in comparison with x-rays across the entire pion beam path.

(c) Extension of fixed geometry techniques to fractionation studies. Systematic experiments employing multiple fractions will be initiated to determine the optimum compromise between repair in the plateau and relatively less repair in the peak region.

(d) Studies to confirm whether cells exposed in the low-LET plateau region of the pion beam will exhibit considerable cycle-dependent variability in radiosensitivity, and whether cells exposed in the peak region will exhibit a uniformly high radiosensitivity.

(2) Animal Radiobiology and Pathology

(a) Early initiation of studies of late effects of high-LET radiation on normal animal tissues (brain, spinal cord, heart, lung, gut, and kidney).

(b) Studies of acute and late effects on skin (using pigs, rats, and mice)

(c) Measurements of the clonogenic potential of cells in situ in irradiated mice, to provide early RBE estimates for designing late effects experiments with fast neutrons and negative pions and to provide in vivo confirmation of RBE measurements derived from cell-culture systems. These studies will examine spleen colony formation by bone marrow stem cells and regeneration of intestinal crypt cells after irradiation.

(d) Tumor radiobiology and control achieved by peak and plateau negative pions in comparison with responses to conventional irradiation (using tumor transplants and spontaneous tumors).

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

Date

Principal Investigator

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DETAILED PROGRESS REPORT

(M.M. Kligerman, L. Rosen, and G.L. Voelz)

1. Objectives

The primary objective of this project is to provide, as soon as possible, information crucial to the radiotherapist for beginning clinical trials of pion radiation therapy for patients with advanced cancer. Specific objectives are:

a. To develop, test, and apply methods of pion dosimetry that are essential to the radiobiology experiments and which will logically carry over into the early pilot trials and subsequent clinical trials of the efficacy of pion therapy for cancer patients.

b. To employ appropriate quantitative cellular radiobiology techniques to establish the relative biological effectiveness (RBE), oxygen enhancement ratio (OER) and effects of dose fractionation of pions under radiation conditions and geometries most informative to the radiotherapist, and

c. To observe in appropriate experimental animals early and late responses and radiopathology of normal and tumor tissues exposed to various segments of the pion beam path to assess acute effects and delayed sequelae as a function of dose and exposure conditions.

Because of the complexity of the project, specific goals for each of the three project years were not established in the original application. To a large degree the activities encompassed under the above objectives are intended to converge over the course of the three-year period toward amassing a sufficient amount of knowledge to enable the start of human trials of pion radiation therapy and to address crucial problem areas that may arise after the start of human trials.

It should be pointed out, however, that the progress under this grant is contingent to a large degree upon progress in program components outside the realm of this grant (for example, the completion of the pion biomedical facility and activation of the pion biomedical beam, both being funded by the Atomic Energy Commission, rather than the National Cancer Institute).

Pions were first obtained at Los Alamos on 26 August 1973, and considerable progress has been made in tuning and dosimetry associated with the main channel which will carry over into operation of the biomedical channel. The biomedical channel is expected to be activated on 26 January 1974, and completion of the biomedical facility is anticipated in April 1974. Although initiation of radiobiology studies with the pion beam awaits the activation of the biomedical channel, considerable headway has

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been made in establishing baseline data for cell systems and animal tissue (both normal and tumorous), using 250 KVP x-rays, which will then be compared with acute and long-term results achieved with negative pi mesons of varying intensities, in plateau and peak dose ranges. The progress to date is described in the following section.

2. Current Studies

This section describes studies conducted at Los Alamos Scientific Laboratory (LASL) and the University of New Mexico Cancer Research and Treatment Center since the start of the grant period on 30 June 1973 to approximately 15 January 1974. Full-scale studies were not authorized to begin at Los Alamos until 1 September 1973, due to delay resulting from negotiation and subsequent approval by the U.S. Atomic Energy Commission and Department of Health, Education, and Welfare of a contract between the University of New Mexico and the University of California (which operates LASL].

a. Biomedical Beam Operation

(1) Tuning and Dosimetry (E.A. Knapp, J. Helland, M.A. Paciotti, A. Lundy, M. Minor, S. Shlaer, J.E. Barnes, J. DiCello, R. Hutson, O. Rivera, and C. George). The magnetic channel that collects, purifies, and directs on the patient the beam of negative pions for therapy is now complete enough to start the tuning with a pion beam, and all components of the channel should be installed during the next shutdown of the LAMPF accelerator which is scheduled for March 1974. The tuning of this beam line is one of the activities funded under this grant for preclinical studies for pion radiotherapy. Such tuning is a complex physics experiment in its own right, where apparatus is developed to trace the trajectory of pions through the magnet system to allow the precise adjustment of the magnetic field values to optimize the flux and spatial distribution of pions in the stopping region within the target volume. The apparatus being constructed for this tuning is a combination of multiwire proportional counters and scintillation counters, reading the trajectory information directly into a control computer which analyzes the information and presents the particle rays pictorially to the experimenter. This apparatus is now constructed and being checked out in preparation to tuning the channel in the next few weeks.

Development of the computer control system for the biomedical beam channel is progressing satisfactorily. All computer hardware is now installed in the control room of the facility, and all factory adjustments have been made. A complex executive program which directs the computer installation in its activities has been installed and is being tested at the present time. The programs that allow operator interaction with the multitude of tasks which the computer will accomplish, such as the oscilloscope display programs, printer programs, magnet control programs, and data collection programs, are mostly developed at the present time and will be used in the initial tuneup of the beam line and in the initial dosimetry experiments.

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Equipment for the dosimetry required to begin initial biological experiments on the pion beam is under development. Conventional ion chamber systems will be used initially for mapping the depth-dose characteristics of the beam, and these are on hand and have been checked out a low level. A high activity cesium-137 source has been acquired and will be used to calibrate the ion chambers in the near future. Proportional counter ion chambers are being fabricated to investigate the LET distribution in various parts of the beam. A gas handling system for these counters has been fabricated, and an ion chamber laboratory has been set up for these calibrations and other developmental work. We are planning on using solid state detectors for investigation of the LET distributions also. These systems have been set up in the laboratory, and satisfactory spectra using radioactive sources have been obtained.

(2) Computerized Pion Treatment Planning and Port Visualization (J.E. Barnes and W.J. Shlaer). A comprehensive treatment planning program has been developed which will allow the depth-dose distribution to be calculated for various pion beam arrangements in combinations of tissue, bone, air space, and other density inhomogeneities in the patient. This program now operates on a large CDC 7600 computer but is being transcribed to operate on the control computer in the biomedical channel control room. This operation will be possible simultaneously with the control function of the channel.

Three-dimensional calculations can be made for any combination of pion beams. Corrections are made for inhomogeneities and surface curvature. Provisions are made for weighting beams and for including an RBE for the star-products. Output from the code is in the form of two-dimensional isodose maps for planes at any angle through the treatment volume. A number of sample treatment plans have been calculated using the code and it promises to be a very useful tool. Additional check-out of the code will include comparison of calculations to measured dose distributions. An instruction manual for use of the code has been written and was recently published by LASL.

Visualization of the pion treatment port and correlation of the treatment volume with the patient's anatomy present different problems with pions than with conventional radiation therapy radiation sources. A technique for making a film record of the pion port treated and an equally magnified radiograph of the patient's anatomy is presently being developed. The films for recording the pion field and the anatomy would be placed above the patient and a diagnostic x-ray tube would be located below the patient. A paper describing the technique is presently being written.

b. Cellular and Mammalian Radiobiology

(1) Spatial Distribution of Mammalian Cell Lethality (M.R. Raju, D.F. Petersen, R.A. Walters, and T.T. Trujillo). Further significant developments in methods for irradiating cultured mammalian cells in three-dimensional configurations include the adoption of the gelatin method of Skarsgard and Palcic.[1] Cells were suspended in gelatin in 8 x 200-mm

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plastic tubes and irradiated either end-on or side-on with 250-KVP x-rays. Gels were extruded from the tubes, sliced into equal lengths and incubated at 37°C to soften the gel. Cell attachment and colony development could then proceed in tissue culture dishes without further disturbance of the cells. In all cases, cell survival (based on colony formation) followed that predicted from the x-ray beam profile and depth-dose profile. It is clear that this will be one of the methods of choice in pion irradiations of cultured cells.

Other methods of cell holding that have been investigated include culture in agar using a modification of Fischer's method[2] and allowing colonies to develop in situ before the gel is sliced for scoring. The murine lymphoma lines S/S L5178Y and S/S L5178Y and the mastocytoma P-815Y have been grown successfully by this procedure with virtually quantitative plating efficiencies. Crude relative biological effectiveness (RBE) values for lymphoid tissue can be obtained from these lines, and the low D_0 values, particularly for S/S L5178Y, make extremely attractive material for early negative pion experiments in view of the low total doses required. Because these systems require no manipulation between seeding and scoring, they may be of value as models for fractionation experiments.

A study has also been made of our ability to hold cells at lower than normal temperatures for extended time periods--a condition anticipated in early pion irradiations. It has been found that the plating efficiency of CHO cells remains within 95% of control values for at least 48 hours when held in dilute suspension in growth medium at 4°C. In this respect, CHO is superior to the other lines to be employed in these studies, and its capacity for prolonged holding at low temperatures without loss of viability will permit adequate dose accumulation at the low dose rates expected during early phases of accelerator operation. Survival experiments employing these conditions probably will be the earliest biological experiments performed with the low-intensity pion beam.

(2) Effect of Mixed Radiations on Cell Survival in the Presence and Absence of Oxygen (M.R. Raju, J.H. Jett, and T.T. Trujillo). Cultured mammalian cells have been irradiated with various mixtures of alpha particles from plutonium-239 and 250-KVP x-rays. In general agreement with predictions of Hall[3] who used californium neutrons, results to date indicate that small (<20%) admixtures of alpha particles lead to small enhancements of cell killing but to very significant reductions in oxygen enhancement ratio (OER). A complete plot of RBE and OER versus percent alpha particle dose has been generated, and it is anticipated that this set of data will considerably enhance our understanding of pion action when various mixtures of stopping and in-flight pions are used in biological irradiations. It is of particular interest to understand the effects of different radiation mixtures, as the portion of stopping pions will be inversely related to the treatment volume. This study also aids in understanding the reduced OER found in high-energy helium ion irradiations reported previously.[4]

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(3) Cell-Cycle Dependence of Survival at High Linear Energy Transfer (M.R. Raju, R.A. Tobey, J.H. Jett, and T.T. Trujillo). Chinese hamster cells (CHO) were synchronized by mitotic selection and irradiated with 250-KVP x-rays or plutonium alpha particles at hourly intervals throughout the cell cycle. At two different doses, colony-formation survival was independent of cycle state following alpha particle irradiation and was strongly dependent upon cycle stage following x-irradiation. The observations are in substantial agreement with those of Bird and Burki,[5] but indicate that the alpha particles used by Hall et al.[6] may not have had sufficiently high linear energy transfer (LET) to produce a cycle-independent response. It is of even greater interest to assess the age response of cultured human cells (e.g., human kidney T-1, which have been studied extensively with high-LET radiations), and progress is being made in obtaining synchronous cultures of these cells.

(4) Early and Late Effects in the Acutely Irradiated Mouse Foot (M.R. Raju, R.F. Archuleta, and L.M. Holland). Erythema and desquamation of the skin of the mouse paw and loss of nails and toes have been used by Field[7] to assess early and late effects, respectively. This system is being adopted for early use in the pion studies since results can be obtained rapidly. A preliminary set of experiments has been performed in which paws of mice were irradiated with 250-KVP x-rays and graded for erythema and desquamation by a traditional graded scale. A dose-effect curve which satisfactorily resembled that of Field et al.[8] for single exposures was obtained. The animals were observed for the development of late effects which were also graded on a deformity scale devised by Field.[7] Again the dose-response curve was substantially the same as that previously reported, and we consider that this system is adequately calibrated in our laboratory for pion studies in the very near future. Studies using fractionated exposures will also be initiated. In view of the strategic position occupied by skin in the tissue RBE hierarchy of patients treated with neutrons,[8] and the convincing evidence for close correspondence between experimental skin systems, these may be the most useful measurements for establishing early patient exposure conditions.

(5) KHT Tumor Regression with Fractionated Doses of X-Rays (M.R. Raju and R.F. Archuleta). A series of single-dose experiments has already been completed in which skin reactions and tumor regrowth were evaluated in KHT sarcoma-bearing mice. Consultation with Dr. R.F. Kallman of Stanford University has been very helpful in this work. The KHT tumor has been found to be a useful, quantitative model, owing to its reliable growth rate in spherical shape. Irradiations have also been delivered (250-KVP x-rays) at three fractions per week, and it was found that the tumor doubling time following a given total dose was shorter, as expected, when the dose was fractionated. It is customary to irradiate early (small) tumors, and a mouse holder has been designed and tested so that geometrical misses will be avoided in the future when this system is tested using the pion beam.

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(6) Development of the EMT Mouse Tumor System for Pion Radiobiology (M.R. Raju, S.G. Carpenter, T.T. Trujillo, J.E. London, and L.M. Holland). Experience of others performing pretherapeutic radiobiology studies indicates that a cell system which can be readily used for tumor formation *in vivo* and for colony formation *in vitro* is a very useful tool. The EMT mouse tumor cell line developed by Rockwell and Kallman[9] has been chosen for our studies with pions, as it will also be used in similar studies at Stanford. It is possible to irradiate these cells *in vivo* or *in vitro* under hypoxic or oxygenated conditions and to assay them for viability *in vivo* or *in vitro* using tumor formation (100 days) or colony formation (10 days) as the respective end points. The tumor cell line has now been growing successfully *in vivo* and *in vitro* for about one year (we are grateful to Dr. Kallman for providing us with a starter culture and advice). An experiment has been performed in which EMT tumor cells were irradiated in culture, in living mice, and in recently killed mice, with this last condition defining the anoxic state. *In vitro* (colony formation) survival curves were obtained and were typical of fully oxygenated, mixed, and fully anoxic populations, respectively. This work is significant in that the LAMPF Biomedical Steering Committee recommended that the mammalian system OER (if one is to be determined) be found for cells irradiated *in vivo*, where the anoxic state is more readily achieved and defined. In preparation for more extensive studies with pions, x-ray experiments with this cell line are continuing.

(7) Radiobiology of Vicia Faba Root Meristems (M.R. Raju, O.S. Johnson, R.F. Archuleta, and J.F. Spalding). The radiation sensitivity of the root growth rate in seedlings of the broad bean *Vicia faba* makes it ideal for studies of radiation effects at moderate and low doses, and its reputation for reliably measuring OER in neutron[10] and pion[11,12] beams has already been established. Holders have been built, and x-ray exposures in the presence and absence of oxygen have been performed using both the traditional British strain and locally grown strains of Vicia, and the anticipated results were obtained. This system is ready for pion experiments at any time.

(8) Preliminary Studies for the Pion Biomedical Program (L.M. Holland, E.L. Gillette, J.R. Prine, S.G. Carpenter, J.E. London, and R.F. Archuleta). Studies underway include work with three transplantable mouse tumors, skin studies using two models, and ancillary work with jig development. An experiment to establish the tumor control dose-50 (TCD-50) for the C3H mammary carcinoma has been started. The tumor is being studied utilizing 250-KVP x-rays under hypoxic, ambient, and hyperbaric conditions using 60 animals per group. A second study with a fractionated exposure scheme is in its initial stages.

Preliminary skin experiments with 250-KVP x-rays using pigs have begun. Dose effects can be studied with up to six dose patches per animal. An investigation of the effect of pigment on the response of skin to radiation is planned for this spring.

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Several prototype animal holding jigs have been developed which offer the capability of exposing various numbers and sizes of animals. Jigs and shields have been built for a wide variety of organ exposures (skin, lung, urinary bladder, kidney, and various gut segments), and chambers for hyperbaric studies of tumor-bearing mice have been developed. Blood gas studies have been performed on mice to determine the effects of anesthesia and confinement on blood oxygen and carbon dioxide levels.

(9) Ultrastructural Pathology of Irradiated Kidney--Late Effects (S. Jordan, C. Key, M. Holland, T. Phillips, and J. Agnew). A number of investigators, including Luxton and Kunkler,[13] Madrazo, et al,[14] and Phillips and Benak,[15] have described the renal morphology occurring several months after therapeutic doses of ionizing radiation, which is associated with compromised renal function and systemic hypertension and which is clinically limiting to the therapist. The changes described as radiation nephritis consist of progressive arteriolonephrosclerosis and renal tubular atrophy, the latter perhaps secondary to the small vessel changes. Sclerosis of arterioles, capillaries and venules occur prominently after irradiation in kidneys as well as in other tissues.

Three questions are being addressed with respect to the morphologic changes following renal irradiation:

(a) What light and electron microscopic changes occur and at what time intervals after irradiation?

(b) What is the RBE of pion vs. neutron vs. conventional irradiation causing a given degree of pathologic alteration? Pions and neutrons have not yet been available for this work, which has been done so far with conventional 250-KV x-ray.

(c) Can terminal structure and/or function of kidney be predicted relatively quickly after irradiation from its morphologic appearance?

Young adult LAF_1/J male mice were subjected to unilateral nephrectomy (right-sided) and were allowed to stabilize for four weeks before irradiation of the remaining kidney with a well-shielded 250-KV conventional x-ray source. The remaining hypertrophied kidney was located by palpation and a lead cylinder shield put into position over it. Single dose localized kidney exposures were administered to each of 10 randomly selected unilaterally nephrectomized animals at each of the following dose levels: 0, 1250, 1500, 1750, 2000, and 2250 rads. Groups of animals were irradiated August 3, 1972 and September 11, 1972 using facilities at the Los Alamos Scientific Laboratory. Animals have been sacrificed according to the following schedule:

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	0	1250	1500	1750	2000	2250
0 Time	10	-	-	-	-	-
2 months	-	1	-	1	-	1
3 months	3	1	1	1	1	1
4 months	1	1	1	1	1	1
6 months	2	2	2	2	2	2
9 months	-	2	2	1	2	2
12 months	1	2	1	2	1	2

Currently, light and electron microscopic evaluation of renal histologic changes are being carried out on tissue from the sacrificed animals. As of this writing in January 1974, only two animals have spontaneously died during the course of the experiment. The remaining animals feed regularly, are fat, and seem quite healthy.

More animals will be sacrificed at 18 months after irradiation and the remainder no later than 24 months after irradiation. We plan to repeat the irradiation with 250-KV x-ray at higher doses in view of the paucity of changes seen to date, and then begin pion and perhaps neutron evaluation as these modalities become available.

3. Significance

A promising approach to improvement of radiotherapy is through the application of high-LET radiations. Negative pions constitute a new and untried high-LET radiation with additional potential advantages relative to depth-dose distribution, low-LET in intervening normal tissue, finite range, sophisticated exposure control, and other aspects.

The significance of this project is derived from the construction at LASL of a negative pion beam many times more intense than any in existence or being planned anywhere in the world. The investigations of physical and biological properties of pions being conducted under this project are an essential prerequisite to pilot studies and subsequent controlled clinical trials of pion radiotherapy for cancer patients.

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It is the intent of this project to develop the technology to establish whether negative pions are indeed as promising as present knowledge suggests. If pions offer a significant advancement in radiotherapy and the ultimate in cancer control has not been achieved, much of the pion therapy technology will have been developed, and development of accelerators to provide negative pion beams in major cancer therapy centers for treatment of large numbers of patients at acceptable cost can go forward promptly and with confidence.

4. Research Goals for Year 2

a. Biomedical Beam Operation

While the major portion of effort associated with initial tuning of the pion beam is expected to be completed by the start of Year 2, it should be pointed out that this process will need to receive continual attention during the course of the project to achieve the refinements necessary to support the proposed biology experiments and ultimately the use of the beam for cancer patient therapy.

During the coming year work will continue on the treatment planning code. Improvement of the beam model to correlate with measured dose distributions will be necessary. The computer code presently runs on a CDC 7600 computer at the Los Alamos Computing Center. It is planned that modifications will be made to the code to allow it to be run in a more interactive mode on the PDP 11/45 computer at the Biomedical Facility.

Dosimetry studies scheduled to begin as soon as the pion biomedical channel is activated include development of reliable ion chambers for the channel, measurement of depth dose and lineary energy transfer (LET) distributions in a water phantom, and later in more realistic phantoms, measurement of neutron dose outside the treatment volume due to the star fragments, development of the pion stopping region visualization apparatus, and development of reliable computer hardware and software for dosimetric purposes.

b. Cellular and Mammalian Radiobiology

Work in those areas detailed in Section 2 will continue, with the initiation of phases of each experiment involving pions contingent on beam intensity. Thus, priorities have been arranged to reflect both the importance of the information and the practical aspects of efficiently utulizing a beam that will increase in intensity from a few rads per minute to full intensity over a period of 12 to 24 months. Segments of the study not mentioned in Section 2 will be initiated as soon as practical. For example, those segments to be performed by consultants represent extensions of ongoing programs to be conducted on-site as soon as appropriate beam intensities are available.

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C1 Cellular Radiobiology

(a) RBE measurements. The initial experiments are designed to provide short-term RBE values; the most important single parameter required for exploratory patient work is the determination of the RBE of pions in the plateau and peak regions of beam to confirm theoretical predictions. These experiments will employ two widely used and well characterized mammalian cell lines: Chinese hamster (line CHO) and human kidney T-1 cells. The former line has been used extensively for single-cell survival studies employing low-LET radiation. The line is tolerant to shifts in temperature and oxygen tension, has a well characterized cell cycle, can be synchronized easily to a high degree, and perhaps most important has a high and reproducible plating efficiency. Human T-1 cells have been employed extensively in studies using high-LET radiation in several laboratories,[16, 17] and were used in the preliminary studies with negative pions at the Berkeley cyclotron.[18] The initial studies will deal with establishment of reliable RBE values as a function of depth in tissue-equivalent material.

(b) Oxygen enhancement ratio. One of the most attractive features of negative pion therapy is that much of the dose delivered to a treatment volume is densely ionizing and, as a consequence, has a higher efficiency for killing hypoxic tumor cells than low-LET radiation employed in conventional therapy. At the same time, normal tissue proximal to the treatment volume is exposed at low-LET, affording amplification of the difference in biological effects in favor of the patient. The greater the proportion of hypoxic cells in a tumor, the more important this advantage becomes; therefore, it is of interest to obtain quantitative estimates of the oxygen enhancement ratio (OER) in comparison with x-rays across the entire pion beam path.

(c) Dose fractionation. Stabilization of mastocytoma cell suspensions in viscous medium has been investigated and will permit extension of fixed geometry techniques to fractionation studies. Such a technique has the advantage that cells remain in register with respect to the LET distribution along the beam path and would incorporate features of cell-cell interactions on survival not considered in conventional fractionation techniques. By the time negative pions become available, the utility of these procedures will be established and, if they are successful, will contribute materially to studies with fractionated pion exposures. A first approximation will involve an arbitrary 48-hour fractionation schedule in a split-dose experiment to measure repair capacity at various depths in the beam profile. Having achieved this goal, systematic experiments employing multiple fractions will be initiated to determine the optimum compromise between repair in the plateau and relatively less repair in the peak region.

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(d) Cycle-dependent radiosensitivity. Although large quantitative differences in cycle-dependent radiosensitivity have been reported (depending upon the cell system employed), a general feature exhibited by cells in vitro appears to be a peak of radioresistance coincident with the latter part of S phase which has dimensions approaching the magnitude of the effect observed in hypoxic cells exposed to high doses of low-LET radiations. With lower doses of low-LET radiation, as well as high-LET radiations, the cycle-dependent sensitivity tends to be diminished, and the response is cycle-dependent. Hence, a biphasic response is anticipated in cells exposed to pions, depending upon their position in the beam path, and experiments to establish this fact are of interest from the standpoint of dose fractionation.

Chinese hamster cells (line CHO) can be synchronized conveniently to a high degree (95% of the population in a 10-minute window in the last third of metaphase) and in adequate yield by mitotic selection. Synchronized cells will be collected and allowed to traverse in conventional suspension culture at 37°C. At frequent intervals corresponding to subfractions of the major cycle phases, aliquots of appropriate size will be removed, suspended in viscous medium, and exposed either to the pion beam or to 250-KVP x-rays. Coincident with each exposure, an aliquot of the stock suspension culture will be subjected to a cell-cycle distribution measurement to assess the proportion of cells in each of the cycle phases to correct for age dispersion which occurs unaviodably during cycle traverse. The microfluorometric cell-cycle analysis method involves measurement of the genome content of individual cells and thus provides a direct estimate of fraction of the population in G_1, S, and G_2 + M. By combining these techniques, accurate cycle-dependent radiosensitivity profiles can be constructed spanning the entire cell cycle in terms of age and the pion beam in terms of changing LET. The amount of information potentially available from this experimental design is large because other variables (for example, the degree of oxygenation and dose rate) can be introduced systematically to provide additional useful data for planning tumor treatment schedules.

The expectation is that, despite considerable cycle-dependent variability in radiosensitivity of cells exposed in the low-LET plateau region of the negative pion beam, cells exposed in the peak region will exhibit a uniformly high radiosensitivity. Current explanations of tumor cell radioresistance under conditions of fractionated exposure invoke redistribution of cells to a relatively resistant cycle phase between exposures to low-LET radiation. The reduction of cycle-dependent radioresistance to high-LET radiation could be one of the advantages of negative pion therapy; extensive understanding of the response could play an important role in optimizing fractionation schedules.

(2) Animal Radiobiology and Pathology

One of the highest priorities is attached to the early initiation of studies of late effects on normal tissues. Few established end points for the quantitative assessment of late effects exist, and the experiments must rely heavily on functional observations and histopathological examination. Animals showing symptoms or scheduled for

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necropsy will be systematically examined for microscopic changes in brain, cord, eye, heart, lung, liver, kidney, gut, skin, bone, and bone marrow as time permits. As these studies develop, attempts will be made to quantitate findings either by analytical or cytologic means.

A significant problem in evaluation of morphologic effects occurring in tissue after irradiation is the objective quantitation of morphologic changes. Such quantitative evaluation should be more sophisticated than an all-or-none (alive-or-dead, necrosis-or-no necrosis) end point or the perforce subjective 1+, 2+, 3+, 4+ of classical pathology. Parameters of potential usefulness for assessing radiation changes include such measurements as quantity and distribution of fibrosis and the wall thickness, diameter, and number of capillaries per unit cross-section of tissue. Cytologic measurements of interest include absolute cell and nuclear sizes multinucleation, nucleocytoplasmic ratio, and distribution characteristics of these cell measurements in a population of irradiated cells (e.g., epithelial cells exfoliated from irradiated urinary bladder). Fibrosis and vascular cross-section may be useful in predicting viability or ability to repair damage of irradiated tissue; cytologic changes may be a useful comparison of relative biological effectiveness (RBE) between x-rays and negative pions. All the measurements mentioned above can be done by various conventional means, principally by the Chalkley point-count technique and determination of absolute and relative areas by planimeter or by cutting out and weighing regions of photographic prints. However, these techniques are slow and tedious, which limits the number of individual determinations and hence the statistical validity of any results.

(a) Late effects of high-LET radiation in normal tissues

Brain, spinal cord, heart, lung, gut, and kidney. A potential advantage of negative pion therapy stems from the fact that large tumors may be treated intensively with more discrete energy deposition than is the case for conventional technique or fast neutrons. In fact, this ability may be the major advantage of negative pions in therapy, but the limited information on normal tissue effects (e.g., late vascular and connective tissue changes and/or extensive necrosis) and the uncertainty of fractionation effects on RBE must be determined experimentally prior to a clinical trial. Available information has been gained largely from therapy experience. Studies of late effects of high-LET radiations are crucial, particularly in view of (1) the uncertainty of current impressions concerning radiosensitivity of the heart and (2) the potentially disastrous effects of misjudging the influence of fractionation on the RBE of fast neutrons and negative pions.

Adult Sprague-Dawley rats and New Zealand rabbits will be exposed by direct anterior field to x-rays or collimated 14-MeV neutrons. Thus, the 1.5-cm field will contain the heart, portions of lung, and a segment of thoracic spine. A similar field in another series will be directed laterally to the head. Single doses ranging from 2500 to 6400 rads in 300-rad increments (15 animals per dose) will be administered, and the animals will be examined weekly for development of neurological effects. Animals exhibiting

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paralysis, ataxia, or loss of sphicter control, and any dying animals will be autopsied for complete histopathological studies. A similar study on the effects of negative pions will be started as soon as reasonable definition of the beam has been accomplished. From these studies late effects will be determined on brain, spinal cord, bone and bone marrow, heart, pericardium, and lung. Similar experimental designs and radiation fields will be used to explore late effects on gut and kidney.

In view of past experience with high-LET radiations, it is crucial that the effects of fractionation on RBE be well understood for each of the tissues mentioned. Thus, as the tissue tolerance doses are established under local radiation conditions, assumptions based on short-term RBE measurements will be used to design fractionation schedules for fast neutrons and negative pions. These schedules will be used to approach the critical question of tissue tolerance and late effects from the standpoint of practical therapy. At the moment, these parameters are sufficiently vague to prevent designing the experiment in detail, but it is clear that the RBE under conditions of fractionated exposure can exceed 2.0 and can vary significantly from tissue to tissue.[19]

Skin. Tissue tolerance in radiotherapy, conventionally gauged by skin tolerance, has recently received increased attention due to renewed interest in fast neutrons in radiotherapy. A clinical trial with pions cannot be conducted without adequate skin response data. However, one of the most compelling arguments for the therapeutic application of negative pions has been the advantageous LET distribution which, in contrast to fast neutrons, should exert effects on skin no greater than those encountered in conventional therapy.

For the acute experiments anesthetized male and female pigs of standard light-skinned breeds (4 to 6 months of age) will be exposed to 250-KVP x-rays and to the plateau and peak regions of the negative pion beam. The Hammersmith 8-patch system, in which each animal serves as its own control,[20] will be used. Recognizing the similarity of LET distributions, the negative pion doses have been chosen on the basis of previous fast neutron data,[21] and experimental designs are intentionally similar to those of the Hammersmith studies to facilitate comparison of negative pions and fast neutrons. The initial acute experiments will involve four animals exposed to similar doses of 1500, 1700, 1900, and 2100 rads of 250-KVP x-rays and to the terminal pion region at the highest available dose rate for the field size used. Skin response will be graded 1 to 5, erythema-to-moist desquamation in over 50% of the field.[20] At each examination the lesions will be recorded photographically with a color reference system, and biopsy material will be taken for microscopic examination when, in the opinion of the independent observers, histopathological documentation is indicated. Impressions of independent observers will be averaged approximately weekly over the time-course of the lesions to establish the acute dose-response. The acute experiment will then be repeated taking into account adjustments in dose to bracket better the dose-response.

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Rat and mouse skin yield essentially the same impressions and exhibit the same phenomena as have been observed following pig skin irradiation.[19] In addition, a much larger number of animals can be conveniently handled so that detailed dose-response curves can be constructed from the observation of a larger population. In addition, functional response of the foot will be studied. Field's study,[19] one of only a few dealing with late effects of high-LET radiations, makes two important points. First, early reactions are insensitive indicators of late effects, which are minimal until a threshold or critical dose is reached and which then increase sharply. Second, once tolerance doses are approached, small increases in dose can result in disastrous overtreatment.

Groups of four to six adult Sprague-Dawley rats and RFM mice will be exposed to acute and daily fractionated doses of 250-KVP x-rays, fast neutrons (7- to 14-MeV), and negative pions in both the plateau and peak regions. Responses will be numerically scored,[19] early and late, by periodic observation. The high end of the dose range will be intentionally extended to doses causing early extensive breakdown and late advanced deformity. Anticipated doses for multiple fractions of negative pions assume an RBE of 2.0 (x-ray doses will be identical with plateau pion doses and twice the doses for fast neutrons and peak pions, but dose ranges will be broad enough to detect an RBE approaching 3.0).

(b) Clonogenic systems for estimating short-term RBE. To provide early RBE estimates for designing late effects experiments with fast neutrons and negative pions and to provide *in vivo* confirmation of the RBE measurements derived from cell-culture systems, the clonogenic potential of cells *in situ* will be measured in irradiated mice.

Spleen colony formation by bone-marrow stem cells. Weanling RF mice exposed to negative pions either in the plateau or pion stopping region or to 250-KVP x-rays will be sacrificed immediately after doses of 100 to 500 rads of x-rays or plateau pions and after doses of 50 to 250 rads of stopped pions administered at maximum dose rates. Known numbers of cells will be injected intravenously into lethally irradiated (about 950 rads) isologous recipients. Eight to 10 days after grafting, the recipients will be sacrificed and spleen colonies scored for both number and histologic classification. Assuming an RBE of 2.0 for stopped pions, minimum survival of marrow stem cells should be 1% or less, and confirmation of the assumption would provide early evidence that the predicted RBE values of 1.0 for plateau pions and 2.0 for stopped pions are indeed reasonable estimates.

Regeneration of intestinal crypt cells. Compelling evidence that gut death in experimental animals results from loss of stem cells in the intestinal crypts demands that reliable RBE estimates for crypt cells be obtained prior to treatment of pelvic tumors, which unavoidably include intestine in the treatment volume. For these studies histologic examination of transverse sections of mouse jejunum will be used to assess regeneration of crypt stem cells, as well as histopathologic changes in other tissues

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included in the radiation field. The mouse is small enough to be given whole-body radiation either in the low- or high-LET regions of the pion beam for comparison with animals exposed to 250-KVP x-rays and 14-MeV neutrons by the microcolony assay of Withers and Elkind.[22] Approximately 3.5 days after whole-body irradiation, RF mice will be killed, segments of jejunum will be fixed and stained, and the number of regenerating crypt cells will be calculated on the basis of Poisson statistics from the expectation of 80 to 100 crypts per circumference in unirradiated controls. It is obvious that this system lends itself well to dose fractionation studies, which will be started when the acute RBE values for low- and high-LET regions of the negative pion beam have been established.

(c) Tumor radiobiology and control. For a number of spontaneous and transplantable tumors, radiobiological parameters (e.g., D_0, hypoxic fraction, tumor control dose (TCD-50), and capacity for reoxygenation) are known in some detail for conventional radiations and for fast neutrons. In the initial stages of the present investigation, the observations of interest are comparative: What are the effects of peak and plateau negative pions relative either to known responses from the literature or to measured responses employing conventional radiation?

Transplantable tumors. A suitable transplantable tumor system is the C3H mammary carcinoma described by Suit and Urano.[23] An inoculum containing approximately 10^5 viable cells will be injected into the gastrocnemius muscle of the C3H mouse and allowed to grow to a diameter of 8 mm (2 weeks). The tumors will be irradiated with 250-KVP x-rays and plateau and peak negative pions and observed for 120 days. During that period, desquamation, fibrosis, and mobility of the leg will be noted. The end point is local tumor control. A tumor which regrows to 8 mm or larger is scored as recurrence; a failure to regrow to the original diameter in 120 days is scored as a cure. Intercomparison of the four radiations at varying doses will indicate whether tumor control is achieved with fewer complications by stopped pions (that is, the TCD-50 or the dose required to achieve local control in half the treated tumors is directly comparable for each modality, and the observed complication dose (CD-50) for desquamation, loss of mobility, etc., obviously will vary with LET). Hence, the combination of local control with degree of complication is directly accessible in this system.

Spontaneous tumors. For testing therapy regimes under practical conditions, experimental treatment in dogs of spontaneous tumors which are slow-growing, exist under natural conditions of immunosurveillance, and have counterparts in human neoplasia has been suggested by the Radiotherapy Subcommittee. For these studies, a referral arrangement will be established with the small animal practice of the area in order to ensure sufficient clinical material. From a dog population in the surrounding area of approximately 1,000,000 animals, 4000 diagnosed tumors can be expected annually. Twenty-five percent of the tumors would be of potential experimental interest and, in principle, 100 cases of a specific histologic classification (e.g., squamous cell carcinoma or fibrosarcoma of the head and neck) could be assembled for a systematic comparison of negative pions with conventional technique. Those spontaneous tumors of sufficient interest to pursue would be subjected to a typical patient workup and confirmatory diagnosis,

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including histopathologic classification and identification of coexisting complications. Finally, on a random basis, tumors of similar classification would be treated with optimum conventional technique or pions. Evaluation of results should provide the most direct comparison of the therapeutic efficacy of negative pions for a variety of major tumor classifications.

In the coming year, much of the work in this area will be related to establishing the logistics of the referral system. Limited large-animal holding facilities are now available. Initiation of exposures is contingent on beam intensity and priorities described in this document for Year 2.

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