DOE/ER/60719--T1

Oliver W. Press M.D. Ph.D. March 24, 1992

OHER Nuclear Medicine Research Project Assessment

1. Project Title:

Improved Radioimmunotherapy of Hematologic Malignancies

DOE/ER/60719--T1

DE92 012504

RPIS #:	9913
DOE Grant #:	DE-FG06-88ER60719
Principal Investigator:	Oliver W. Press MD PhD
Organization:	University of Washington
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2. Patentable Inventions: Not Applicable

3. Principal Project Personnel:

I. Oliver W. Press (Principal Investigator)

A. Role in Project: Dr. Press designs and analyzes all experiments, is responsible for the project's overall supervision as well as preparation of manuscripts, abstracts, oral presentations and grant renewals.

B. Principal Areas of Research and Expertise: Dr. Press has an M.D., a Ph.D. in Biological Structure/Cellular Immunology, and is board-certified in Internal Medicine and Medical Oncology. He has been engaged in biomedical research for the past nineteen years, including eight years working with antibody conjugates.

C. Percent Effort: Dr. Press currently spends >30% of his time on this project, but because of budgetary constraints, is receiving only 20% salary from this project.

D. Education:

B.S. in Biology, Stanford University, 1973.
Ph.D. in Biological Structure, University of Washington, 1977.
M.D., University of Washington, 1979.
Internship and Residency in Internal Medicine, Massachusetts General Hospital, Boston, 1979-82.
Chief Residency in Medicine, University of Washington, 1982-83.
Fellowship in Oncology, University of Washington, 1983-84.

E. Relevant Professional Employment History:

1) Clinical Fellow in Medicine, Harvard Medical School (1979-1982).
2) Acting Instructor in Medicine, University of Washington (1982-1986)
3) Assistant Member, Fred Hutchinson Cancer Research Center, Seattle 1986-1991)
4) Assistant Professor in Medicine, University of Washington (1987-1991)
5) Adjunct Assistant Professor of Biological Structure (1988-91)
6) Adjunct Associate Professor of Biological Structure (1991-present)
7) Associate Member, Fred Hutchinson Cancer Research Center (1991-present)
8) Associate Professor in Medicine, University of Washington, (1991-present)

F. Relevant Honors:

1) George E. Gamble Honors Scholarship (Stanford U., 1970-1973)
2) Phi Beta Kappa (1973)
3) Departmental Honors and Departmental Distinction in Biology (Stanford, 1973)
4) Alpha Omega Alpha, Honorary Medical Fraternity (elected as 3rd year student in 1976)
5) Scholarship from International College of Surgeons (1978)
6) PhD Thesis Honors (U.W. 1979)
7) Medical Auxiliary M.D. Ph.D. Award (1979)
8) Graduation with "Highest Honors" from U. of Washington School of Medicine (1979)
9) Robert S. Evans Award (1979)
10) John J. Bonica Prize in Anesthesiology (1979)
11) Lamport Biomedical Research Prize (1979)
12) Seattle Academy of Internal Medicine Prize (1979)
13) Young Investigator Award from the American Society for Clinical Oncology (1985-1986)
14) Clinical Oncology Career Development Award from the American Cancer Society (1985-1988)
15) First Independent Research and Training Award from the NIH (1987-1992)
16) Elsa Pardee Award for Cancer Research (1988-1989)

G. Recent Relevant Publications Not Emanating From this Project
(Grant-related publications are listed on Pages 17-19).

1. Press OW, Appelbaum F, Ledbetter JA, Martin PJ, Zarling J, Kidd P, and Thomas ED. Monoclonal antibody 1F5 (anti-CD20) serotherapy of human B cell lymphomas. Blood 69: 584-591, 1987.

2. Press OW, Martin PJ, Thorpe PE, and Vitetta ES. Ricin A-chain containing immunotoxins directed against different epitopes on the CD2 molecule differ in their ability to kill normal and malignant T cells. J. Immunol. 141: 4410-4417, 1988.

3. Press OW, Mortimer J, Collins C, and Livingston R. "Oncologic Therapeutics", In Medical Therapeutics, (Larson EB and Ramsey PG, eds.), W.B. Saunders Co., pp. 297-358, 1989.

4. Press OW. Immunotoxins. Biotherapy 3: 65-76, 1991.

5. Press OW, Livingston RB, Mortimer J, Collins C, and Appelbaum FR. Treatment of relapsed non-Hodgkin's lymphomas with dexamethasone, high dose cytarabine, and cisplatin (DHAP) prior to marrow transplantation. J. Clin. Oncol. 9: 423-431, 1991.

6. Press OW. "Lymphadenopathy". In Medical Diagnostics (D.C. Dugdale and M. Eisenberg, eds.), W.B. Saunders Co., (In press), 1992.

7. Press O. A commentary on combined chemoradiotherapy for the treatment of Hodgkin's disease. Oncology (In press), 1992.

8. Martin PJ, Hansen JA, Torok-Storb B, Moretti L, Press O, Storb R, Thomas ED, Weiden PL, Uhr JW, and Vitetta ES. Effects of treating marrow with a CD3-specific immunotoxin for prevention of acute graft versus host disease. Bone Marrow Transplantation. 3: 437-444, 1988.

9. Appelbaum F, Badger C, Bernstein I, Bianco J, Brown P, Eary J, Press O, Sandmaier B, Schuening F, and Storb R. Development of improved marrow transplant preparative regimens: Use of antibody-radionuclide conjugates, In Technical Proceedings of the ACNP-SNM Joint Symposium on Biology of Radionuclide Therapy, (G.L. DeNardo, ed.), American College of Nuclear Physicians, Washington, D.C., pp. 102-109, 1989.

10. Bernstein ID,Eary JE, Badger CC, Press OW, Appelbaum FR, Martin PJ, Krohn KA, Nelp WB, Porter B, Fisher D, Miller RA, Brown S, Levy R, and Thomas ED. High dose radiolabeled antibody therapy of lymphoma. Cancer Res. 50: 1017-1021, 1990.

11. Petersen FB, Appelbaum FR, Press OW, et. al. Autologous marrow transplantation for malignant lymphoma. J. Clin. Oncol 8: 638-647, 1990.

12. Eary J, Press O, Badger C, Martin P, Appelbaum F, Krohn K, Levy R, Brown S, Miller R, Fisher D, Nelp W, and Bernstein I. Imaging and treatment of B cell lymphoma. J. Nucl. Med. 31: 1257-1268, 1990.

13. Appelbaum FR, Badger CC, Eary JF, Matthews DC, Press OW, and Bernstein ID. Therapy of hematologic malignancies using radioimmunotherapy (RAIT). Cambridge Medical Reviews: Haematology-Oncology, Vol. 2. (In press), 1992.

14. Appelbaum FR, Matthews D, Eary JF, Badger CC, Kellogg M, Press OW, Martin PJ, Fisher D, Nelp WB, Thomas ED, and Bernstein ID. Use of radiolabeled anti-CD33 antibody to augment marrow irradiation prior to marrow transplantation for acute myelogenous leukemia. Transplantation (In press), 1992.

4. Additional Project Personnel:

I. Susan Kay Anderson (Senior Research Technologist)

A. Role in Project: Ms. Anderson is a full time research associate who spends 100% of her time performing the laboratory experiments described here. Her list of duties include preparation, purification, and radiolabeling of monoclonal antibodies, maintenance of tissue culture cell lines, performance of cellular radioimmunoassays, SDS PAGE gels, electron microscopy, thin layer chromatography, autoradiography, and flow cytometry. Her full time participation is essential to the success of this project.

B. Principal Areas of Research and Expertise: Susan has 15 years of laboratory experience performing the techniques mentioned above.

C. Percent Effort: 100%

D. Education: B.A., University of Oregon (Eugene), 1975.
M.S., University of Oregon (Eugene), 1976.

II. Aykut Bilge M.D.

A. Role in Project: Dr. Bilge has recently joined our lab on a full time basis and will continue the studies of the intracellular routing and catabolism of immunoconjugates which were formerly done by Drs. Geissler and DeSantes (see below).

B. Principal Areas of Research and Expertise: Dr. Bilge has five years of laboratory experience in the fields of microbiology and tumor immunology.

C. Percent Effort: 100%

D. Education:

M.D. Hacettepe University School of Medicine, Ankara, 1982.
Residency in Microbiology, Gazi University, Ankara, Turkey 1986-90.
Ph.D. candidate in Biological Structure, U. of Washington, 1989-present.

III. Prasanna Venkatesan

A. Role in Project: Consultant

B. Principal Areas of Research and Expertise: Dr. Venkatesan is a radiochemist in the Division of Nuclear Medicine at the University of Washington with extensive experience in radioimmunoconjugate synthesis (including ^{131}I-tyramine celloboise and ^{186}Rhenium conjugations). Dr. Venkatesan and Dr. Press have been working together on the ^{131}I-tyramine celloboise for the past year, and have planned the described experiments with the radiometal chelates (especially ^{186}Rhenium). He works under the supervision of Drs. Nelp and Dr. Kenneth Krohn.

C. Education and Experience:

B.S. in Chemistry, Vivekananda College, Madras, India, 1968.
M.S. in Chemistry, Indian Inst. of Tech. Madras, India, 1970.
Senior Radiochemist, Radiochemistry Division, Bhabha Atomic Research Center, Trombay, Bombay, India, 1971-78.
Ph.D. in Chemistry, Penn. State University, 1984.
Research Affiliate in Nuclear Engineering, MIT, Boston, 1984-1987.
Research Associate, Harvard Medical School, Boston, 1986-87
Radiochemist, NeoRx Corporation, Seattle, 1987-90.
Research Assistant Professor in Nuclear Medicine, U. W. , 1990-present.

IV. Wil B. Nelp M.D.

A. Role in Project: Consultant

B. Principal Areas of Research and Expertise: Dr. Nelp has been the Head of the Division of Nuclear Medicine at the University of Washington for the past 29 years, and has published extensively on the radioimmunodetection and radioimmunotherapy of human malignancies. Dr. Press and Dr. Nelp have collaborated for five years on both clinical and laboratory radioimmunoconjugate projects.

C. Education and Experience:

B.S. in Chemistry, Franklin College of Indiana, 1951.
M.D., Johns Hopkins School of Medicine, 1955.
Internship and Residency, Johns Hopkins, 1955-57, 1959-60.
Research Fellow in Medicine and Radiology, NIH Nuclear Medicine Department, 1960-62.
Professor of Medicine, Radiology, & Nuclear Medicine, U.W. , 1971-92
Head of the Division of Nuclear Medicine, 1962-present .

VI. Irwin Bernstein M.D.

A. Role in Project: Consultant

B. Principal Areas of Research and Expertise: Dr. Bernstein is the Head of the Division of Pediatric Hematology-Oncology at the University of Washington and the Fred Hutchinson Cancer Research Center and is the Principal Investigator for the U.W./FHCRC phase I clinical trials of radioimmunotherapy for hematologic malignancies. Dr. Bernstein is an expert on hematopoiesis and has synthesized numerous monoclonal antibodies targeting human lymphoid and myeloid malignancies (e.g., MoAb P67-6).

C. Education:

B.S. in Biology, Trinity College, Hartford, Connecticut, 1963.
M.D., New York University, 1967.
Pediatric Intern and Resident, Children's Hospital, Los Angeles, 1967-69.
Fellow in Pediatric Hematology-Oncology, U. of Washington, 1971-73.
Fellow, New York Cancer Research Institute, 1972-74.
Professor of Pediatric Hematology-Oncology, UW, 1981-present.
Member of the Fred Hutchinson Cancer Research Center, 1981-present.
Head of the Division of Pediatric Hematology-Oncology, 1978-present.

VII. Christopher Badger M.D.

A. Role in Project: Consultant

B. Principal Areas of Research and Expertise: Dr. Badger is an an expert on the experimental radioimmunotherapy of lymphoid malignancies in murine models, on the pharmacokinetics of radiolabeled antibodies after IV administration, and is a key participant in our collaborative human trials of radioimmunotherapy.

C. Education:

B.S. in Biology, Brown University, 1973.
M.D., Brown University, 1976.
Medical Resident, Brown University Affiliated Hospitals, 1976-79.
Senior Fellow in Medical Oncology, University of Washington, 1979-82.
Acting Instructor in Medicine at the University of Washington, 1982-present.
Assistant Member, Fred Hutchinson Cancer Research Center, 1982-present.

VII. Kenneth B. De Santes (Postdoctoral Fellow)

A. Role in Project: Dr. De Santes was a postdoctoral fellowship for two years performing cellular radioimmunoassays, radioimmunoscintigraphy, and radioimmunotherapy experiments for the Her2/neu oncogene project (1989-91).

B. Principal Areas of Research and Expertise: Dr. DeSantes had primary responsibility for the targeting of breast and ovarian xenografts with radiolabeled antibodies recognizing the Her-2/neu oncoprotein (Aim 5). Unfortunately, because of restrictions in the revised DOE budget and a delay in the delivery of funds for this project, it was no longer possible to pay Dr. De Santes' salary, and he has recently assumed a position at UCSF.

C. Percent Effort: 100% in 1989-91; 0% in future

D. Education:

B.S. in Biology, State University of New York at Albany, 1979.
M.S. in Immunology, University of Michigan, 1980.

M.D., New York Medical College, 1984.
Residency at Children's Hospital of Los Angeles, 1984-1987.
Fellowship at Children's Hospital and Medical Center, Seattle, 1987-90.

IV. Francis Geissler
A. Role in Project: Graduate Student 1988-90, Post-doctoral fellow 1990-91.
B. Principal Areas of Research and Expertise: Dr. Geissler studied the endocytosis, intracellular routing, and catabolism of radiolabeled antibodies by malignant T and B lymphoma cell lines.
C. Percent Effort: 100% in 1988-90.
D. Education:
B.S. in Biology, University of Washington, 1983.
M.S. in Environmental Health, University of Washington, 1986.
Ph.D. in Biological Structure, University of Washington, 1990.

5. Project Overview

A. Overall Objective: This research project proposes to develop novel new approaches of improving the radioimmunodetection and radioimmunotherapy of malignancies by augmenting retention of radioimmunoconjugates by tumor cells. The approaches shown to be effective in these laboratory experiments will subsequently be incorporated into our ongoing clinical trials in patients.

B. Specific Project Objectives

1. To study the rates of endocytosis, intracellular routing, and metabolic degradation of radiolabeled monoclonal antibodies targeting tumor-associated antigens on human leukemia and lymphoma cells. From 1988-91 we systematically studied the behavior of a large panel of MoAbs recognizing the major surface antigens present on malignant T lymphoid, B lymphoid and myeloid cell lines. In 1992 we began studies using fresh malignant cells obtained from patients with malignant B and T cell lymphomas and myeloid leukemias seen at the University of Washington and the Fred Hutchinson Cancer Research Center.

2. To examine the effects of lysosomotropic amines (e.g. chloroquine, amantadine), carboxylic ionophores (monensin, nigericin), and thioamides (propylthiouracil), on the retention of radiolabeled MoAbs by tumor cells. In studies done from 1988 to 1991 we established the capability of these reagents to double the retention of radioiodinated antibodies by tumor cells in vitro, and determined the maximally tolerated doses of these agents in rodents. In 1992 we plan to examine the effects of maximally tolerated doses of these agents on radioimmunoscintigraphic and radioimmunotherapeutic efficacy in an *in vivo* tumor xenograft system.

3. To examine the impact of newer radioiodination techniques (tyramine cellobiose, paraiodobenzoyl) on the metabolic degradation of radioiodinated antibodies. In vitro experiments conducted from 1988 to 1991 have established that tyramine cellobiose constructs resist intracellular metabolic degradation and afford superior tumor retention of radioimmunoconjugates compared with conventional iodination techniques (chloramine T or IodoGen). In the future we plan to analyze the intracellular extent of degradation of tyramine cellobiose constructs in more detail and analyze in vivo the merits of these novel constructs for radioimmunoscintigraphy and radioimmunotherapy.

4. To compare the endocytosis, intracellular routing, and degradation of radioimmunoconjugates prepared with different radionuclides (^{131}Iodine, ^{111}Indium, ^{90}Yttrium, ^{99m}Technetium, ^{186}Rhenium). Preliminary studies indicate that many radiometals are retained intracellularly after radioimmunoconjugate catabolism, in contrast to ^{131}I which is rapidly exocytosed from cells after metabolism of conventional (Iodo-Gen, chloramine T-labeled) ^{131}I-RAbs.

5. To examine the utility of radioimmunoconjugates targeting oncogene products for the radioimmunotherapy and radioimmunoscintigraphy of cancer. Special emphasis will be placed on studying the efficacy of MoAbs targeting the Her2/neu oncoprotein expressed on the surface of poor prognosis breast and ovarian cancers.

B. Relationship to the DOE Nuclear Medicine Program mission.

This project promises to improve the efficacy of radioimmunotherapy for treatment of cancer, the second leading cause of death in the United States. Therefore, it directly addresses the Human Health and Assessments research goals of the Office of Health and Environmental Research enunciated in the Federal Register 50: 14867: "to develop new techniques for stable and radioactive isotope production, labeled pharmaceuticals, imaging devices, and radiation beam applications for the improved diagnosis and therapy of human diseases".

Budget removed. ds

C. Relationship to other projects funded by DOE.

The findings of this research study should have important implications for other DOE-funded radioimmunotherapy and imaging studies. (We are not privy to the list of other DOE grant awardees).

6. Scientific and Technical Content

A. Relation of this Research to the Field of Radioimmunotherapy: Monoclonal antibodies (MoAbs) directed against tumor-associated antigens and conjugated to toxins, drugs, or radionuclides afford an attractive mechanism by which tumoricidal agents might be selectively focused on malignant cells while sparing normal cells from the toxic influence of these agents (1-3). Of the antibody-conjugate approaches, radioimmunoconjugates have yielded the most impressive results in early clinical trials (4-8). In Seattle, we have administered high dose radioimmunotherapy with ^{131}I-labeled anti-B cell antibodies to 17 patients with Non-Hodgkin's Lymphomas who failed

treatment with conventional chemotherapy and radiotherapy. All 17 of these refractory patients achieved tumor regressions, including fourteen patients who achieved complete disappearance of all tumor, two who achieved partial responses (>50% tumor disappearance) and one who experienced a minor response (40% shrinkage). Despite these impressive results in refractory patients, it must be admitted that most other clinical trials have not been as dramatic, and even in our study most patients have subsequently relapsed (10 of 17). Furthermore, 14 other patients evaluated for our treatment protocol were rejected because preliminary trace-labeled antibody infusions demonstrated rapid RAb catabolism and/or inadequate tumor localization (based on gamma camera imaging and tumor biopsies). Thus, considerable room for improvement exists in optimizing the efficacy of radioimmunotherapy.

Several factors have been identified which limit the efficacy of radioimmunotherapy as currently practiced, including inadequate penetration of RAbs into tumors, formation of human anti-mouse antibodies, marked myelosuppression by RAbs, nonspecific hepatic uptake of RAbs, and rapid catabolism of RAbs *in vivo* (particularly "dehalogenation" of radioiodinated MoAbs). The goal of this proposal is to investigate methods of circumventing the last problem listed, and to delineate strategies which enhance retention of RAbs by tumor cells, thereby enhancing the tumor: normal tissue ratios of absorbed radiation. We have focused on tumoral catabolism of RAbs because it has been a pervasive problem in clinical studies with lymphomas and leukemias both at our institution and elsewhere (9-18), and because recent research suggests that approaches for mitigating this problem now exist (19-22).

A full comprehension of the behavior of antibody-conjugates after binding to tumor cell surface membranes is required to rationally investigate methods of circumventing RAb catabolism. Many MoAbs targeting solid tumors (e.g. colon cancer) remain statically fixed to antigens of the extracellular glycocalyx after binding. In contrast, most MoAbs targeting lymphoid and myeloid antigens are rapidly internalized by cells after binding, either by receptor-mediated endocytosis or constitutive endocytosis (14-15). In either case, antigen-MoAb complexes are internalized through microinvaginations of the surface membrane into endosomes, and ultimately lysosomes where enzymatic degradation rapidly ensues, resulting in release of free ^{131}I and ^{131}I-tyrosine to the interstitial fluid. Since the efficacy of radioimmunotherapy depends on the specific concentration of RAbs in tumor cells integrated over time, rapid expulsion of ^{131}I is undesirable. We hypothesize that methods which prolong the retention of RAbs by tumor cells should generate improved radioimmunoscintigraphic and radioimmunotherapeutic results.

Four strategies for enhancing tumor retention of RAbs will be addressed by this proposal:

1) Identification of MoAbs which undergo endocytosis slowly and are minimally degraded.

2) Administration of pharmacologic agents which retard catabolism of RAbs at tumor sites.

3) Implementation of novel conjugation techniques (e.g. tyramine cellobiose, paraiodobenzoyl) which produce "non-metabolizable" radioiodinated constructs.

4) Utilization of alternative radionuclides (e.g. ^{111}Indium, ^{90}Yttrium, ^{186}Rhenium) which may be retained intracellularly even after RAb metabolism occurs in tumor cells.

We have deliberately focused the majority of our studies on hematologic malignancies because 1) the surface antigens of lymphocytes and myeloid cells are well delineated, 2) multiple high quality MoAbs are available, 3) the best results of clinical trials with unmodified antibodies and with RAbs have been observed with hematologic malignancies, 4) leukemias and lymphomas are exquisitely sensitive to radiation therapy, and 5) patients with lymphomas are much less likely to form human anti-mouse antibodies than patients with solid tumors. Nevertheless, in the next funding period we

propose to extend our investigations to include breast and ovarian carcinomas in order to test the hypothesis that targeting the Her2/neu oncogene on the surface of poor prognosis breast and ovarian carcinomas will provide effective salvage therapy for these cancers (23). Preliminary results show that rapid RAb catabolism is also a problem with breast cancer cells targeted with ^{131}I-anti-Her2/neu antibodies and that the same therapeutic considerations discussed above will apply to this tumor system (24).

F. Major Recent Research Accomplishments.

1. Studies on the Endocytosis and Degradation Rates of RAbs. We have systematically studied the endocytosis, intracellular routing, and metabolism of a large panel of MoAbs recognizing the major surface antigens present on T and B lymphomas as well as selected antibodies targeting myeloid leukemia cells and breast cancer cells using cellular radioimmunoassays, immunoelectron microscopy, autoradiography, SDS-PAGE electrophoresis, and thin layer chromatography (14,15,19, 22,24-28) These experiments have documented marked variability in the rates of endocytosis and catabolism of ^{125}I-labeled MoAbs as summarized in Table I:

Table I: Characteristics of ^{125}I-Labeled MoAbs Studied

Tumor Line	MoAb (Isotype)	Endocytosis Rate*	% Degraded in 24 Hr*	Antigen Target	# Binding Sites/Cell	Avidity (L/M)
RAMOS (B cell lymphoma)	DA4-4 (IgG1)	Fast	35-50%	IgM	330,000	4.3×10^9
	HD37 (IgG1)	Fast	20-30%	CD19	100,000	3.0×10^8
	HD6 (IgG1)	Intermediate	22%	CD22	90,000	2.9×10^9
	MB-1 (IgG1)	Intermediate	15%	CD37	235,000	3.0×10^9
	7.2 (IgG2a)	Slow	<10%	HLA DR	390,000	1.7×10^9
	B1 (IgG2a)	Slow	<10%	CD20	305,000	3.7×10^8
	BC8 (IgG1)	Slow	<10%	CD45	200,000	5×10^8
HPB-ALL (T cell lymphoma)	64.1 (IgG2a)	Fast	35-60%	CD3	100,000	2×10^9
	35.1 (IgG2a)	Fast	50%	CD2	20,000	1.5×10^9
	66.1 (IgG2a)	Intermediate	25%	CD4	60,000	0.7×10^9
	66.2 (IgG2a)	Slow	<10%	CD8	90,000	2.2×10^9
	BC8 (IgG1)	Slow	<10%	CD45	N.D.	N.D.
HEL (Myeloid Leukemia)	P67-6 (IgG1)	Fast	50-60%	CD33	10,000	1×10^{10}
	BC8 (IgG1)	Slow	<10%	CD45	200,000	5×10^8
AKR/A (Murine T Lymphoma)	OX7 (IgG1)	Slow	<10%	Thy 1.1	1,000,000	7×10^8
SKBR3 (Breast CA)	4D5 (IgG1)	Fast	30-55%	Her2/neu	105,000	1.6×10^9

*Kinetics of internalization and degradation are published in references (14,15,22,24-28).

A typical kinetic profile quantifying the % of RAb present on the cell surface, inside cells, and in the culture supernatant as a function of time is shown in Fig. 1 for ^{125}I-labeled anti-CD3 MoAb 64.1 targeted to the HPB-ALL T cell lymphoma line. In Fig. 2, the radioactivity appearing in supernatants of malignant T cells labeled with ^{125}I-MoAb 64.1 is fractionated into small molecular weight species (<10,000 kD) which are soluble in 25% trichloroacetic acid (TCA), and large molecular weight species which are

TCA-precipitable. It can be seen that early in the culture period (<4 hours), most of the supernatant radioactivity is TCA precipitable (primarily intact RAbs passively dissociated from cell surface antigenic sites), whereas late in the culture period, the majority of radioactivity released from cells is present as small molecular weight species (primarily ^{125}I-monoiodotyrosine as shown by HPLC and TLC).

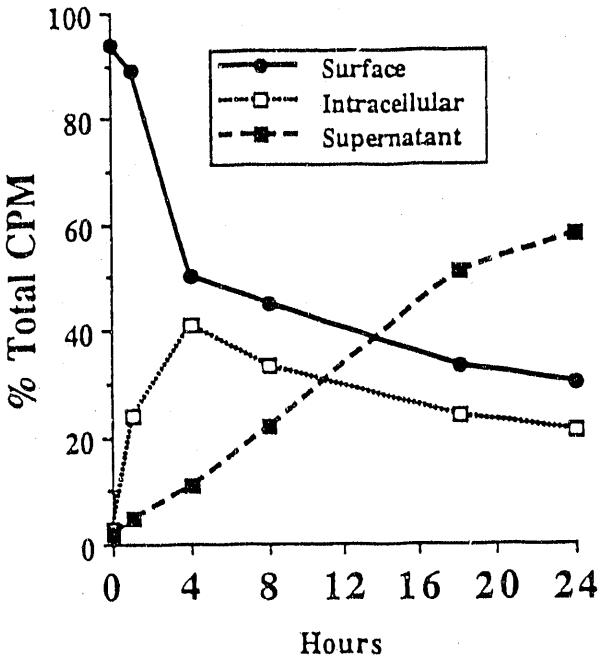

Fig. 1: Distribution of ^{125}I-MoAb 64.1 on the Cell Surface, Intracellularly, and in Culture Supernatants as a Function of Time after Targeting HPB-ALL cells (determined by cellular radioimmunoassay).

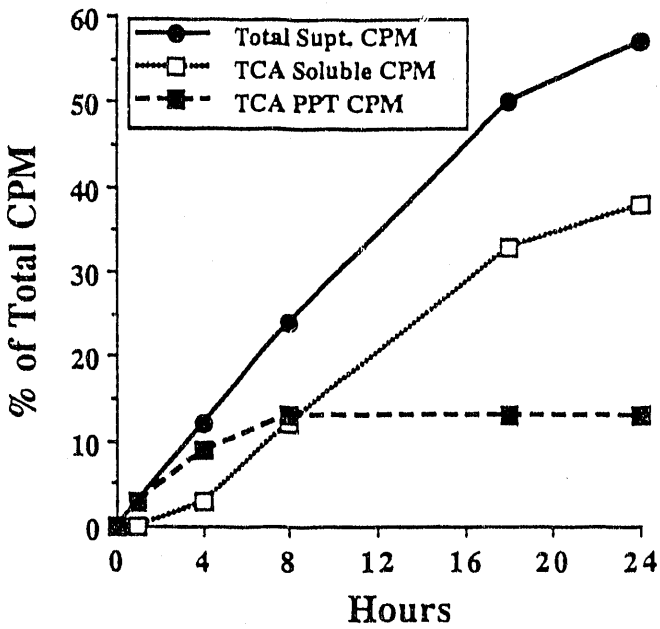

Fig. 2: Accumulation of TCA-soluble, TCA-precipitable, and total cpm in culture supernatants of HPB-ALL cells pulse-labeled at time 0 with ^{125}I-MoAb 64.1.

b. Studies with Metabolically labeled antibodies. Recent studies in our laboratory have shown that antibody internalization and degradation are identical for MoAbs radioiodinated using the IodoGen technique or biosynthetically labeled by incubation of hybridoma cells with ^{3}H-leucine. These data show that the patterns of cellular internalization and degradation for the radioiodinated antibodies faithfully mimic those of native antibody and are not due to protein damage during the iodination process as can occur when harsher iodination conditions are used (25,26). Furthermore, they confirm that lysosomal proteases are more important than "deiodinases" for the catabolism of radiolabeled antibodies.

c. Morphologic documentation of intracellular routing. Immunoperoxidase electron microscopy and ultrastructural autoradiography have been used to directly observe the trafficking of antibody conjugates from the cell surface through clathrin-

coated pits to endosomes and lysosomes (14,15). (Photomicrographs will be shown at the oral presentation).

d. Studies analyzing the trafficking of RAbs through subcellular compartments. Percoll density gradient sedimentation of organelles was used to analyze the intracellular routing of RAbs in tumor cells which had been pulse-labeled with RAbs and then disrupted with a Dounce homogenizer. With this technique, buoyant surface membrane and endosomal fractions (sp. grav. 1.010-1.035) were easily separated from the dense lysosomal fraction (sp. grav. 1.080). The position of organelles on these gradients was documented by marker enzyme analyses (5' nucleotidase and alkaline phosphodiesterase for plasma membrane; ß galactosidase for lysosomes) and by electron microscopy. A typical experiment is shown in Figs. 3 & 4 for HPB-ALL cells pulse-labeled with ^{125}I-MoAb 64.1. At time 0, virtually all ^{125}I-64.1was on the cell surface whereas by 8 hours the majority was present in lysosomes, and by 24 hours all radioactivity had been cleared from the cell surface and the total amount of cell associated radioactivity ("area under the curve") was markedly diminished, presumably due to lysosomal degradation and exocytosis of RAbs.

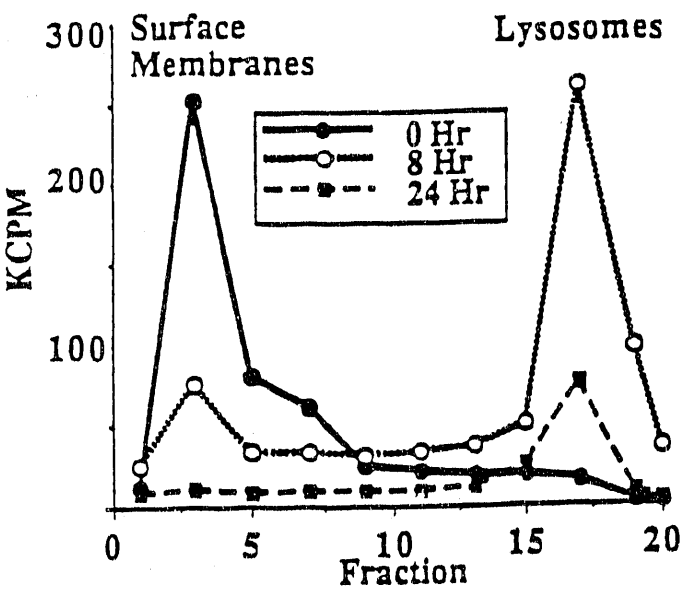

Fig. 3: Transit of ^{125}I-MoAb 64.1 from buoyant (cell surface/endosomal) fractions to dense (lysosomal) fractions on 20% Percoll gradients as a function of time (using HPB-ALL cells).

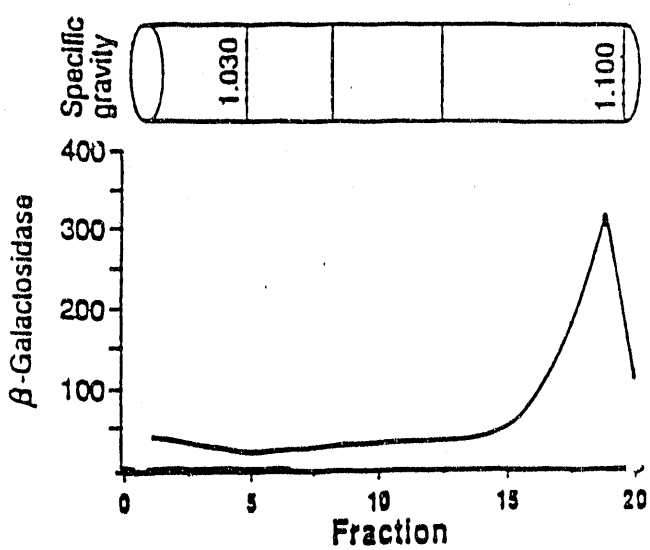

Fig 4: Distribution of the lysosomal enzyme ß-galactosidase on the same 20% Percoll gradient depicted in Figure 7.

e. Biochemical analysis of metabolites. Target cells incubated for various periods of time at 37°C with ^{125}I-labeled RAbs were lysed and analysed by HPLC, SDS-PAGE, and thin layer chromotography (TLC) to determine the sizes of generated fragments. The tempo of RAb degradation was monitored for whole cell lysates, for lysates of organelles purified on Percoll gradients, and for culture supernatants at time intervals ranging from 1-24 hours after pulse-labeling target cells with RAbs. A typical

experiment using ^{125}I-MoAb DA4-4 targeted to RAMOS cells is depicted in the SDS-PAGE and TLC autoradiographs in Figs. 5 & 6. This experiment shows progressive accumulation of intermediate-sized RAb metabolites (50 kD, 28 kD, 25 kD, and 12 kD) inside B lymphoma cells beginning 4 hours after tumor cell targeting. Studies using organelle fractions demonstrated a minimal amount of RAb metabolism in prelysosomal endosomes, with >95% of degradation occurring in the lysosomal fraction. Analysis of culture supernatants revealed that these intermediate sized fragments were not exocytosed from tumor cells in detectable quantities, and that >95% of TCA-soluble supernatant radioactivity (>70% of *total* supernatant radioactivity) was present as ^{125}I-tyrosine. Contrary to popular belief, free ^{125}I represented <10% of culture radioactivity. These results suggest that the process of "dehalogenation" reported by most authors in fact consists primarily of terminal degradation of RAbs to ^{125}I-tyrosine by lysosomal hydrolases rather than liberation of free ^{125}I from RAbs by "deiodinases".

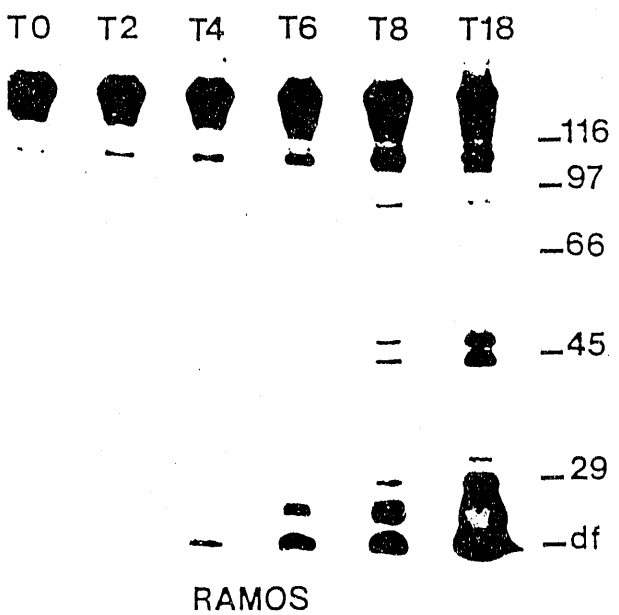

Fig. 5: SDS-PAGE autoradiographic analysis of ^{125}I-labeled-DA4-4 after internalization by RAMOS cells which had been incubated at 37°C for 0,2,4,6,8 or 18 hours. A progressive appearance of metabolites is seen.

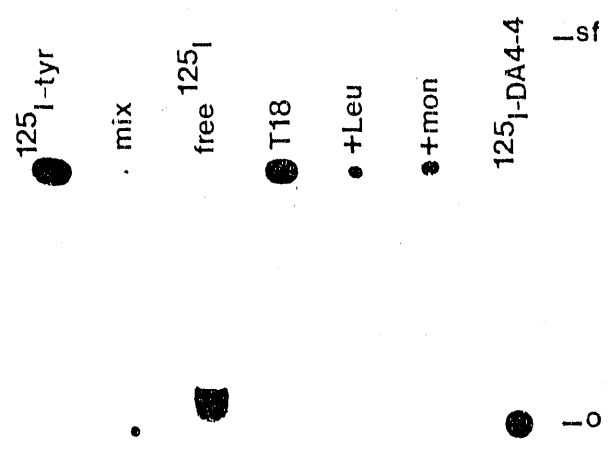

Fig. 6: Thin layer chromatogram of acetone-soluble radioactivity from culture supernatants of RAMOS cells incubated with ^{125}I-DA4-4 for 18 hr at 37°C (lane 4) in the presence or absence of leupeptin (lane 5) or monensin (lane 6). In all circumstances, radioactivity had the same mobility as the ^{125}I-tyrosine standard (lane 1) and not free ^{125}I (lane 3).

In the next funding period, we will confirm that the metabolic processes delineated above for tumor cell lines are also operant in fresh tumors biopsied from patients undergoing clinical radiolabeled antibody therapy at the University of Washington.

2. Studies Documenting the Utility of Pharmacologic Agents for Inhibiting Catabolism of RAbs.

a. In Vitro Studies: A variety of lysosomotropic amines, carboxylic ionophores, thioamides, and calcium channel blockers have been shown to interfere with catabolism of ^{125}I-labeled protein ligands (15). We have tested the hypothesis that these agents can be used to retard degradation of radioimmunoconjugates by tumor cells and augment the retention of RAbs by tumors. The success of this approach *in vitro* is documented in Figs 7 & 8 which demonstrate that the tested drugs decrease the degradation of ^{125}I-64.1 by T lymphoma cells by 15-90% at nontoxic doses (Fig.7) and augment retention of the RAb by 150-330% at 24 hours of culture (Fig. 8)

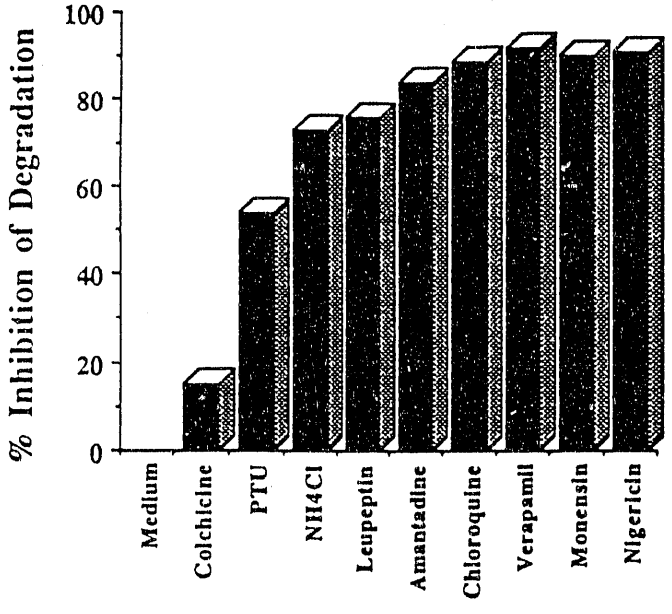

Fig. 7: Inhibition of ^{125}I-MoAb 64.1 Degradation by HPB-ALL cells by 20 μM colchicine, 10 mM propylthiouracil, 20 mM NH_4Cl, 400 μM leupeptin, 2.5 mM amantadine, 0.8 mM chloroquine, 0.64 mM verapamil, 10 μM monensin, and 10 μM nigericin (as determined by appearance of TCA soluble cpm in culture supernatants).

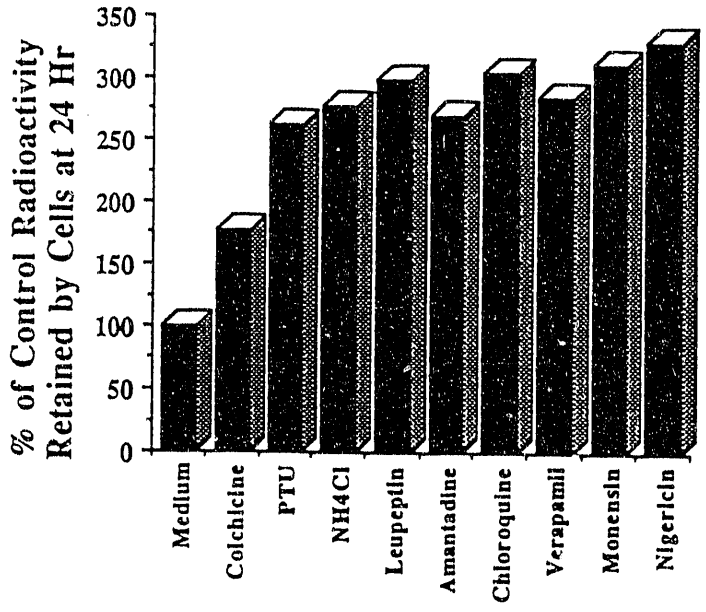

Fig. 8: Augmented Retention of ^{125}I-MoAb 64.1 by HPB-ALL cells after 24 hours of culture with the pharmacologic agents listed in Fig. 5.

b. Assessment of Toxicity of Pharmacologic Agents in vivo. Although the pharmacologic agents investigated work dramatically *in vitro* (Figs. 7 & 8), their effective clinical implementation depends upon whether they can be administered at effective concentrations *in vivo* with tolerable toxicity. To determine the maximally tolerated doses of these drugs in a murine model, escalating concentrations were administered to groups of BALB/c mice (5 each) as outlined in the original grant application. Table II summarizes the highest doses of drugs which could be administered without observing any toxic deaths.

Table II: Maximally Tolerated Doses of Lysosomotropic Agents

Drug	Maximally Tolerated Dose
Single Agents	*by Intraperitoneal Injection*
Monensin	6 mg/kg IP twice daily for 5 days
Chloroquine	70 mg/kg IP twice daily for 5 days
Verapamil	25 mg/kg IP twice daily for 5 days
Propylthiouracil	100 mg/kg IP daily for 7 days
Amantadine	100 mg/kg IP daily for 7 days
Leupeptin	>40 mg/kg IP daily for 7 days
by Osmotic Pump	*by continuous subcutaneous infusion*
Monensin	not feasible--precipitates in pump
Chloroquine	320 mg/kg subQ for 7 days
Verapamil	72 mg/kg sub Q for 7 days
Combinations	*by Intraperitoneal Injection*
Monensin + Chloroquine	6 mg/kg + 20 mg/kg IP twice daily for 2 days
Monensin + Verapamil	6 mg/kg + 12 mg/kg IP twice daily for 2 days

In the next funding period we will examine the ability of these agents to enhance retention of RAbs in tumor xenograft sites, to improve tumor imaging, and to augment radioimmunotherapeutic efficacy.

3. Studies Analyzing New Radioiodination Techniques (e.g. tyramine cellobiose). We have recently shown that novel, new radioiodination techniques using nonmetabolizable aryl carbohydrate adducts (e.g. tyramine cellobiose, tyramine glucose) to link ^{131}I to MoAbs result in prolonged trapping of polar TCB-^{131}I metabolites inside lymphoma cells (19). We have recently confirmed these findings using the anti-CD33 MoAb P67-6, which is used clinically at our institution to target ^{131}I to acute myeloid leukemia (Fig. 9 & 10). Conventional ^{125}I- or ^{131}I-labeled P67-6 RAbs made using the chloramine T or IodoGen methods are rapidly internalized and degraded by leukemia cells, with resultant rapid release of small molecular weight, TCA-soluble metabolites (Fig. 9). In contrast, ^{131}I-TCB-P67-6 constructs are degraded one third as rapidly (Fig. 9) and the amount of cell associated radioactivity after 24 hours of culture is doubled using this novel new method (Fig. 10).

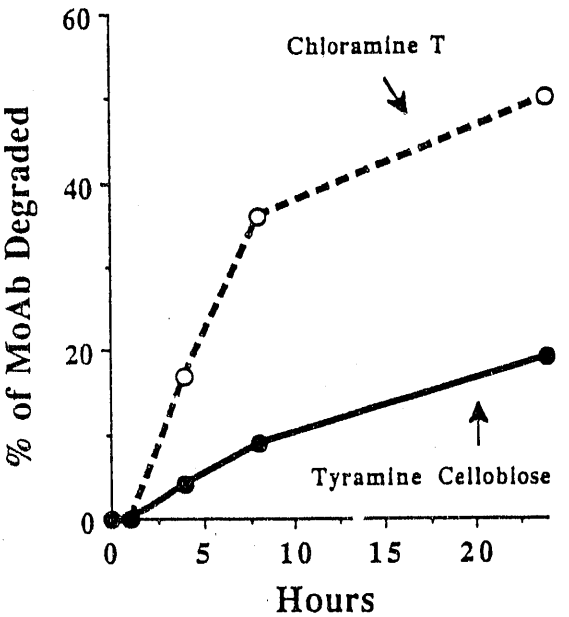

Fig. 9: Diminished rate of degradation of ^{125}I-MoAb P67-6 by HEL cells after conjugation by the tyramine cellobiose method compared with the chloramine T method (as determined by rate of accumulation of TCA soluble cpm in culture supernatants).

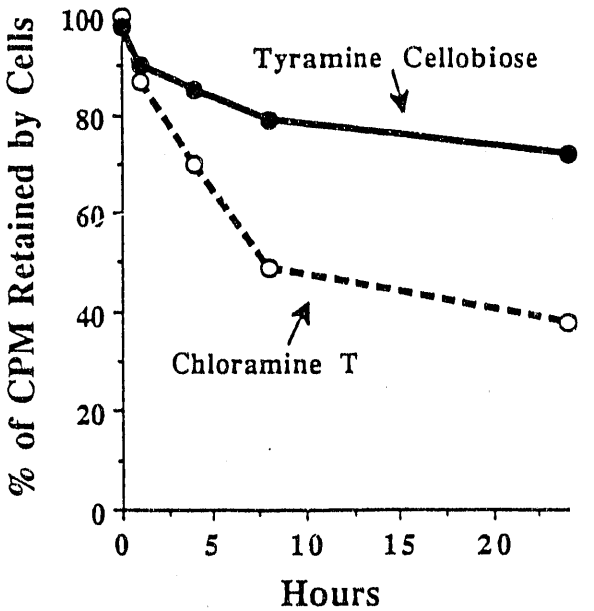

Fig. 10: Enhanced retention of ^{125}I-MoAb P67-6 by HEL cells after conjugation by the tyramine cellobiose method compared with the chloramine T method.

4. Studies Analyzing the Intracellular Routing and Metabolism of RAbs made with radiometals (^{111}Indium, ^{90}Yttrium, ^{99m}Technetium, ^{186}Rhenium). These experiments are just beginning , but will employ identical methodology (cellular radioimmunoassays, SDS-PAGE electrophoresis, thin layer chromatography, HPLC, Percoll gradient fractionation of cell organelles, etc.) to that described above. These experiments will be conducted in conjunction with our collaborators in the Division of Nuclear Medicine (Drs. Nelp and Venkatesan), who have agreed to synthesize and provide the radioimmunoconjugates.

5. Studies Analyzing the Targeting and Radioimmunotherapy of Breast and Ovarian Tumors Using Anti-Her2/neu Antibodies (in collaboration with Dr. Dennis Slamon, UCLA). Cellular radioimmunoassays have shown that breast and ovarian cancer cell lines (SKBR3, SKOV3) and fibroblasts transfected with the HER2/neu gene internalize and degrade anti-HER2/neu antibodies in a manner identical to that described above for our lymphoma studies (24). Experiments in a nude mouse xenograft system have documented the specific localization of injected ^{131}I-4D5 to Her2/neu bearing

tumors (Fig. 11) with tumor to non-tumor ratios of 5-10 to 1 (Fig. 12). Gamma camera imaging has demonstrate excellent tumor localization (Fig.13). Pilot studies have documented th(radioimmunotherapeutic efficacy of this approach (Fig. 14), though all tumors have eventually regrown at the three dose schedules tried so far. Further animal experimentation is required in this new system before its application to refractory human breast cancers can be contemplated.

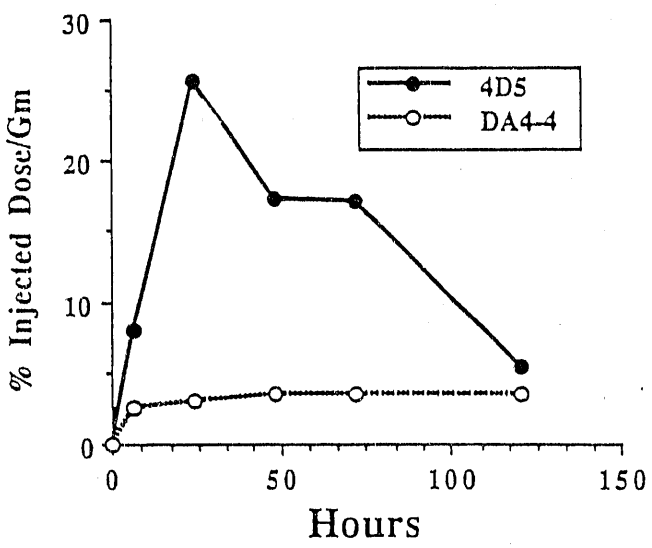

Fig. 11: Localization of ^{131}I-labeled anti-Her2/neu MoAb 4D5 (10 µCi/ 10 µg) and ^{125}I-labeled control MoAb DA4-4 to HER2/neu expressing tumor grafts in nude mice (expressed as % of the injected dose/gram of tumor).

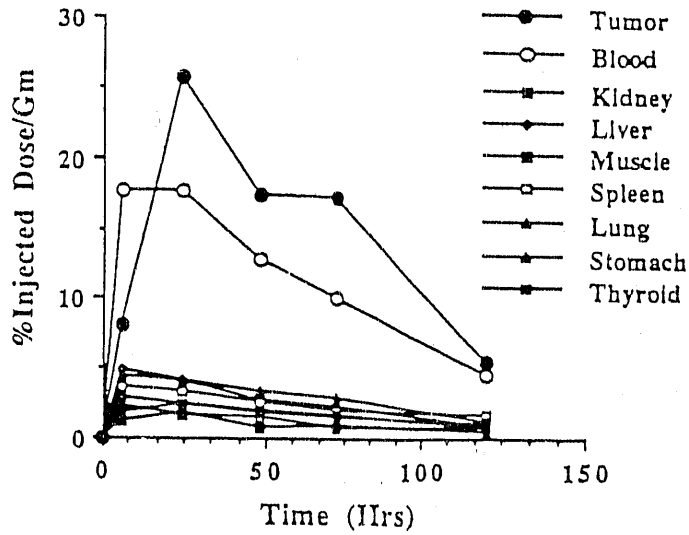

Fig. 12: Biodistribution of ^{131}I-labeled anti-Her2/neu MoAb 4D5 (10 µCi/ 10 µg) in tumor and normal organs as a function of time.

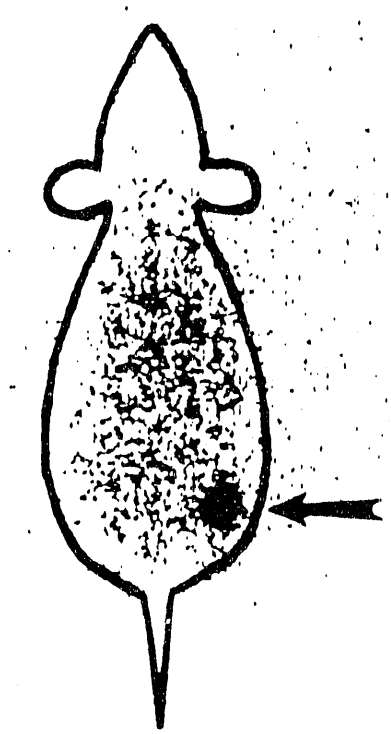

Fig. 13: Tumor imaging with ^{131}I-anti-Her2/neu MoAb 4D5 (400 μCi/ 100 μg) in a nude mouse bearing a subcutaneous HER2/neu-expressing tumor xenograft in the right flank.

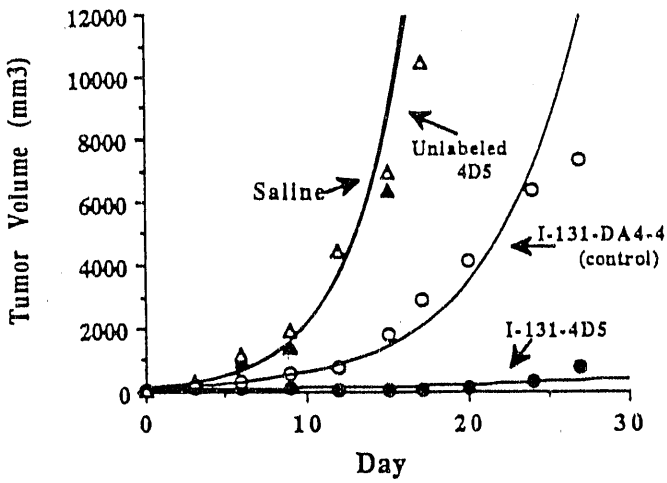

Fig. 14: Inhibition of growth of Her2/neu expressing tumor xenografts in beige/nude mice injected on Day 0 with a single dose of anti-Her2/neu RAb ^{131}I-4D5 (400 μCi/45 μg), ^{131}I-DA4-4 (400 μCi/45 μg of nonbinding MoAb), 45 μg unradiolabeled MoAb 4D5, or saline.

7. Project Output

A. Impact on Clinical Trials. Perhaps the most stringent test of the value of a preclinical, basic science research project such as this one is whether the findings are significant enough to impact on clinical practice. Already, the studies performed in the first three years of this grant have led to three changes in our clinical radioimmunotherapy program at UW/FHCRC:

1. We have switched from using the ^{131}I-anti-CD37 antibody MB1 to the ^{131}I-anti-CD20 antibody B1 to treat patients with B cell lymphomas because the latter has been shown to be less rapidly internalized and degraded (15). This has resulted in a marked improvement in our ability to achieve favorable biodistributions in patients (1 of 17 patients were "favorable" with 2.5 mg/kg of MB1 compared with 9 of 15 patients with 2.5 mg/kg of B1.).

2. We have substituted ^{131}I-anti-CD45 antibody BC8 for ^{131}I-anti-CD33 antibody P67.6 to treat patients with acute myeloid leukemia because the former has been shown to be less rapidly internalized and degraded (18,28). This has resulted in a marked

improvement in our ability to achieve favorable biodistributions in patients (3 of 8 patients were "favorable with 0.05 mg/kg of P67.6 compared with 6 of 7 with the same dose of BC8).

3. We are about to initiate a clinical trial using ^{131}I-anti-CD33 antibody P67.6 synthesized with a tyramine cellobiose adduct to see if this reagent will prove superior to both the chloramine T ^{131}I-P67.6 reagent (because of trapping of radionuclide inside cells), and to ^{131}I-BC8 (because the CD33 antigen is more specific for myeloid leukemias than the CD45 antigen).

B. Publications Resulting from DOE grant DE-FG06-88ER60719:

In the past three years our laboratory has published 16 manuscripts and 7 abstracts. Seven of our manuscripts have been in Cancer Research, two in Blood, one in the Journal of Clinical Oncology, and one in Medical Physics.

1. Press OW, Hansen JA, Farr A, and Martin PJ. Endocytosis and degradation of murine anti-human CD3 monoclonal antibodies. Cancer Research 48: 2249-2257, 1988.

2. Press OW, Eary J, Badger C, Appelbaum FA, Martin PJ, Levy R, Miller R, Brown S, Nelp WB, Krohn KA, Fisher D, DeSantes K, Porter B, Kidd P, Thomas ED, and Bernstein ID. Treatment of patients with refractory non-Hodgkin's lymphomas with radiolabeled MB-1 (anti-CD37) antibody. Journal of Clinical Oncology 7: 1027-1038, 1989.

3. Press OW, Farr A, Borroz I, Anderson S, and Martin PJ. Endocytosis and degradation of monoclonal antibodies targeting human B cell malignancies. Cancer Research 49: 4906-4912, 1989.

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5. Ali SA, Warren SD, Richter KY, Badger CC, Eary JF, Press OW, Krohn KA, Bernstein ID, and Nelp WB. Improving the tumor retention of radioiodinated antibody: aryl carbohydrate adducts. Cancer Research (Suppl.) 50: 783s-788s, 1990.

6. Press OW, DeSantes KD, Anderson SK, and Geissler F. Inhibition of catabolism of radiolabeled antibodies by tumor cells using lysosomotropic amines and carboxylic ionophores. Cancer Research 50: 1243-1250, 1990.

7. Press OW, Eary J, Badger CC, Martin PJ, Appelbaum FR, Nelp WB, Levy R, Miller R, Fisher D, Matthews D, and Bernstein ID. Radiolabeled antibody therapy of human B cell lymphomas. In Advances in Experimental Biology and Medicine "Immunobiology of Proteins and Peptides VI" (M.Z. Atassi, ed.), pp. 91-96. Plenum Press, New York, 1991.

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11. DeSantes K, Slamon D, Anderson SK, Shepard M, Fendly B, Maneval D, and Press OW. Radiolabeled antibody targeting of the HER-2/*neu* oncoprotein. Cancer Research (In press), 1992.

12. Hui TE, Fisher DR, Press O, Eary JF, Weinstein J, Badger C, and Bernstein I. Localized beta dosimetry of ^{131}I-labeled antibodies in follicular lymphoma. Medical Physics 19: 97-104, 1992.

13. Matthews DC, Appelbaum FR, Eary JF, Hui TE, Fisher DR, Martin PJ, Durack L, Nelp WB, Press OW, Badger CC, and Bernstein ID. Radiolabeled anti-CD45 monoclonal antibodies target lymphohematopoietic tissue in the macaque. Blood 78: 1864-1874, 1991.

14. Grossbard M, Press O, Appelbaum F, Bernstein I, and Nadler L. Treatment of hematologic malignancies with antibody conjugates. Blood (In press), 1992.

15. Press OW, Eary J, Appelbaum F, and Bernstein I. "High dose radioimmunotherapy with marrow transplantation". In Immunoconjugate Therapy of Hematologic Malignancies (Steven Rosen, ed.), Kluwer Academic Publishers, (In press), 1992.

16. Press OW, Eary J, Appelbaum F, and Bernstein I. "Radioimmunoconjugate therapy of malignant lymphomas". In Malignant Lymphomas (Bruce Dana, ed.), Kluwer Academic Publishers, (In press), 1992.

Manuscript in Preparation:

17. Venkatesan P, Eary J, Krohn K, Press O, Badger C, Bernstein I, and Nelp W. Enhanced retention of tyramine cellobiose radioimmunoconjugates by tumor cells (in preparation).

Abstracts:

1. Press OW, Eary J, Badger C, Martin PJ, Appelbaum FA, Nelp WB, Krohn KA, Fisher D, Porter B, Thomas ED, Miller R, Brown S, Levy R, and Bernstein ID. "High dose radioimmunotherapy of NonHodgkin's Lymphomas", Proc. San Francisco Cancer Symposium, February, 1989.
2. Press OW, Eary J, Badger C, Martin PJ, Appelbaum FA, Nelp WB, Krohn KA, Fisher D, Porter B, Thomas ED, Miller R, Brown S, Levy R, and Bernstein ID. "Radioimmunotherapy of refractory human malignant B cell lymphomas". Proc. Am. Soc. Clin. Onc. May, 1989.
3. Press OW, De Santes K, Anderson SK, and Geissler F. Inhibition of catabolism of radiolabeled antibodies by tumor cells using lysosomotropic amines and carboxylic ionophores. Antibody Immunoconjugates and Radiopharmaceuticals 3: p. 48, 1990.
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5. Geissler F and Press OW. Endocytosis and degradation of radiolabeled anti-CD3 antibodies by malignant human T cells. Proc. Am. Assoc. for Cancer Res. 31: p. 291, 1990.

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7. Press O, Eary J, Badger C, Appelbaum F, Martin P, Wiseman G, Nelp W, Fisher DR, Miller R, Porter B, Matthews D, and Bernstein I. Radiolabeled antibody (RAb) therapy of relapsed B cell lymphomas. Proc. Am. Assoc. for Cancer Res. (In press), 1992.

Presentations at National Meetings Concerning Research from this Grant:

1. 24th Annual San Francisco Cancer Symposium, (Invited Speaker, February 10-11, 1989).
2. American Society of Clinical Oncology, San Francisco, (Slide Presentation, May 21-23,1989).
3. 5th International Conference on Monoclonal Antibody Immunoconjugates for Cancer, (San Diego, Slide and poster presentations, March 15-17, 1990).
4. 81st meeting of the American Association for Cancer Research, Washington D.C., (Poster presentation, May 23-26, 1990).
5. M.D. Anderson Cancer Center, Houston, TX, (Invited Speaker, October 17, 1990).
6. 6th International Symposium on Immunobiology of Proteins and Peptides, Scottsdale, AZ, (Invited Plenary Speaker, October 26-30, 1990).
7. Keystone Symposium on Monoclonal Antibodies, Denver, Colorado, (Invited Plenary Speaker and Discussion Group leader, March 10-16, 1991).
8. 33rd Annual American Cancer Society Science Writers Convention, (Invited Speaker, March 24-26, 1991).
9. Memorial Sloan Kettering Cancer Center, Invited Slide Talk, December 9, 1991.
10. Keystone Symposium on Bone Marrow Transplantation, Keystone CO. Poster Presentation, January 23, 1992
11. Federation of Associated Societies for Experimental Biology and Medicine, (Invited Talk at Immunology Symposium), Anaheim, April 7, 1992.
12. American Association of Clinical Oncology, Slide Presentation, May 19, 1992.

Graduate Students and Postdoctoral Fellows Trained by this Grant:

1. Dr. Francis Geissler (1988-91)
Thesis: Internalization and Degradation of Radioimmunoconjugates by malignant human lymphoid cells. (Ph.D. awarded: December, 1990.)

2. Dr. Aykut Bilge (1991-present)
Project: Intracellular Routing and Recycling of Immunoconjugates

3. Dr. Walter Trautman (1991-92)
Project: Determinants of Immunoconjugate Efficacy

4. Dr. Kenneth DeSantes (1989-91)
Project: Radiolabeled antibody targeting of the HER-2/neu oncoprotein (1988-191).

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28. van der Jagt RHC, Badger CC, Appelbaum FR, Press OW, Matthews DC, Eary JF, Krohn KA, and Bernstein ID. Cancer Research 52: 89-94, 1992.

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