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POSITRON EMISSION TOMOGRAPHIC IMAGING OF TUMORS USING MONOCLONAL ANTIBODIES

Progress Report

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A. SCOPE AND SPECIFIC AIMS

1. Specific Project Objectives

The overall objective of this research project is to develop methods for utilizing positron emission tomography (PET) to increase the clinical potential of radiolabeled monoclonal antibodies (MAbs). PET is the preferred modality for quantitating the three dimensional distribution of radiotracers *in vivo*. Both diagnostic and therapeutic applications of labeled MAbs could be improved as a result of knowledge obtained through the exploitation of the advantageous imaging characteristics associated with PET. By labeling MAbs with positron-emitting nuclides, it should be possible to quantitate the dynamics of their three-dimensional distribution *in vivo*. Our long-term goals are to apply this approach to investigate the following:

a) **Normal tissue toxicity,** a major factor limiting the utility of radioimmunotherapy. Can PET be utilized to determine more accurately the radiation absorbed dose to critical normal tissues such as bone marrow?

b) **Radiation dose to tumor** which is difficult to determine accurately because of the limitations of conventional imaging technology. Can PET be used to quantitate tumor uptake of radioactivity so that it might be possible to both relate therapeutic response to tumor radiation dose and then attempt to treat only those patients with sufficient tumor uptake? In addition, can tumor uptake be quantitated with sufficient accuracy by PET to permit pre-therapy/post-therapy comparisons to monitor therapeutic response?

c) **Early imaging of tumors.** Clearance of activity from normal tissues following the injection of labeled MAbs generally is slow, necessitating the performance of tumor imaging at least 24 hr after injection. Since the tomographic nature of PET minimizes the contribution of overlying and underlying events to the plane of interest, is it possible to image tumors at much earlier times using this technology?

d) **Evaluation of potential strategies for enhancing MAb uptake.** Approaches for increasing the accumulation of MAb in tumor that are currently being investigated include the use of hyperthermia, external beam irradiation and interferons. Can the ability of PET to quantitate tumor uptake in the living animal be used to facilitate these efforts, allowing each animal (and in the future, each patient) to serve as its own control?

This proposal is concerned with the development of methods for labeling MAbs and their fragments with positron-emitting halogen nuclides. Nuclides under investigation are fluorine-18 and iodine-124. These nulides were selected because of the widespread availability of F-18 and because of our extensive experience in the development of new protein radiohalogenation methods. An additional advantage is the synergism with our research program, funded by the National Cancer Institute (CA

42324), to develop I-131 and At-211 labeled MAbs for radioimmunotherapy.

We have been performing our initial investigations in athymic mouse models using the $F(ab')_2$ fragment of Mel-14, a MAb associated with human gliomas and melanomas. Our group has been working for the past few years on the development of radiolabeled MAbs including Mel-14 for use in the diagnosis and treatment of brain tumors, and we have thus acquired a wealth of background information and methodology pertinent to the experiments outlined in this application.

The research plan of this project remains unchanged from our most recently funded grant proposal and includes the following specific aims:

1. **To develop and optimize methods for labeling MAbs and their fragments with fluorine-18 and iodine-124.** Our goals are to maximize yield, minimize synthesis time (particularly for ^{18}F), maintain MAb affinity and immunoreactivity, and render the procedure suitable for automation.

2. **To examine the tissue distribution of ^{18}F- and ^{124}I-labeled MAbs and fragments in athymic mouse xenograft models.** These experiments provide an indication of stability of label *in vivo* and whether or not specific tumor uptake can be achieved in a time compatible with the nuclide half-life.

3. **To determine the pharmacokinetics of ^{18}F- and ^{124}I-labeled MAbs and fragments in normal dogs using PET.** In addition to demonstrating the safety of these procedures, these studies validate the ability of PET to quantitate normal organ uptake in dogs by comparison with tissue uptake data obtained from the same animals at necropsy.

4. **To examine the tissue distribution of ^{18}F- and ^{124}I-labeled MAbs reactive with canine tumors in dogs with spontaneous cancers.** MAb concentrations in tumors determined by PET are compared with levels determined by tissue sampling. Initial studies will use TP-3 and TP-1 MAbs directed against canine and human osteogenic sarcoma.

5. **To investigate the effect of hyperthermia on the tumor uptake of radiolabeled MAbs and fragments in dogs with spontaneous cancers using PET.** Our results in the athymic mouse model indicate that hyperthermia can increase MAb accumulation in tumor by a factor of two. Using PET, we can pursue these investigations in a more realistic model, letting each animal serve as its own control.

2. Relation to DOE Nuclear Medicine Program Mission

This project attempts to combine the imaging advantages of PET with the potential cellular specificity of monoclonal antibodies. Monoclonal antibody research and PET are two of the areas of research of particular programatic relevance to the DOE Nuclear Medicine Program. Thus, this project should be considered to be very

germane to the DOE Nuclear Medicine Program mission. In addition, the labeling methods developed in this project could, in principle, be adapted for use with smaller amino acid sequence constructs developed by molecular biological techniques, making this work of great relevance to evolving directions of the DOE mission.

The DOE is currently funding a number of projects directed at utilizing radiolabeled monoclonal antibodies for the diagnosis and treatment of a variety of diseases. The methods and approaches developed in this project could be of great value to these programs in that they might facilitate the quantitation of labeled monoclonal antibody distribution and allow earlier detection of cancer, inflammation and damaged myocardium. In addition, the availability of monoclonal antibodies labeled with positron emitters could be used in tandem with metabolic, hemodynamic and receptor-avid tracers for PET being developed in other DOE-funded laboratories.

B. WORK IN PROGRESS

1. Evaluation of ^{18}F-Labeled Antimyosin MAb Fragments in a Canine Myocardial Infarct Model

In myocardial infarction, intracellular myosin is leaked into the extracellular space and is accessible to serum-borne agents. Antimyosin MAb fragments labeled with a positron-emitting nuclide might permit the simultaneous exploitation of antimyosin uptake specificity in damaged myocardium and of imaging advantages and quantitative capabilities inherent in PET. Of the positron-emitting nuclides that are available routinely, fluorine-18 has the longest half-life (1.83 hr) and thus may be of value as a label for MAb fragments. Recently, under the auspecies of this grant, we developed a method for labeling antimyosin fragments with ^{18}F that utilized N-succinimidyl 8-[(4'-[^{18}F]fluorobenzyl)amino]suberate ([^{18}F]SFBS) as the labeled acylation agent. Antimyosin F(ab')$_2$ and Fab fragments could be labeled with ^{18}F with good retention of immunoreactivity. The present study was undertaken in a canine model to determine whether preferential myocardial infarct uptake of ^{18}F-labeled antimyosin fragments could be achieved in a time frame compatible with the half-life of ^{18}F.

Aqueous [^{18}F]fluoride was produced by proton bombardment of [^{18}O]water using a small-volume silver target. The protein acylation agent [^{18}F]SFBS was prepared in three steps. After conversion of aqueous [^{18}F]fluoride ion to tetrabutylammonium [^{18}F]fluoride, 4-[^{18}F]fluorobenzonitrile was prepared by fluoro for nitro exchange in 4-nitrobenzonitrile. Conversion to 4-[^{18}F]fluorobenzylamine was accomplished by treatment of the labeled product with lithium aluminum hydride. Reaction of 4-[^{18}F]fluorobenzylamine with disuccinimidyl suberate for 5 min at room temperature yielded [^{18}F]SFBS. The [^{18}F]SFBS was used either in unpurified form or was purified by high-pressure liquid chromatography (HPLC) using a silica gel column eluted with ethyl acetate.

After evaporation of the organic solvent containing the [^{18}F]SFBS in a glass vial, 1.0-1.1 mg of antimyosin MAb fragment (Fab, 2.7 mg/ml; F(ab')$_2$, 5.6 mg/ml) in

borate buffer (pH 8.5) was added and reacted for 15 min at room temperature. After terminating the reaction by the addition of 0.2 M glycine, the ^{18}F-labeled antimyosin MAb fragment was purified by chromatography over a 1 x 10 cm Sephadex G-25 column eluted with phosphate buffered saline. Protein-associated ^{18}F activity, determined by precipitation with 20% trichloroacetic acid, was between 96-99% for all preparations. In general, about 8 mCi of ^{18}F-labeled antimyosin MAb fragment could be prepared per 100 mCi of [^{18}F]fluoride. A myosin - Sepharose column was used to determine the immunoreactivity of the ^{18}F-labeled antimyosin F(ab')$_2$ and Fab fragments.

Dogs were anesthetized and the left circumflex coronary artery was occluded for 2-3 hr. The occlusion was then released to permit reperfusion and 1-5 mCi of the ^{18}F-labeled MAb fragment was injected 15-60 min later. Serial PET images then were obtained for 2 - 5 hr. Tissue distribution studies were performed on Dogs 1-5 in order to determine whether preferential uptake of ^{18}F-labeled MAb fragments in damaged myocardium could be achieved in a time frame compatible with the short half life of ^{18}F. Normal, border and infarcted regions were determined using triphenyltetrazolium chloride and multiple samples obtained for ^{18}F counting.

In Table 1, the data for infarct:normal myocardium and infarct:blood ratios, as well as the maximum percent injected dose per gram uptake in the infarct are summarized. The highest level of ^{18}F accumulation in infarcted tissue was achieved in animals (Dogs 3 and 5) injected with Fab labeled using HPLC-purified [^{18}F]SFBS. Infarct:normal myocardium tissue uptake ratios for these animals were as high as 11.9:1; however, infarct:blood ratios at 2-3.5 hr were only 1.0 to 1.6:1. Maximum target to nontarget ratios were seen with antimyosin F(ab')$_2$.

Using this fragment, infarct:normal myocardium ratios as high as 20.6:1 and infarct:blood ratios as high as 3.2:1 were achieved. Myocardial tissue uptake data for all samples obtained from Dog 3, injected with the Fab fragment, are shown in Figure 1. In samples from normal myocardium, uptake in endocardium, mid-myocardium and epicardium were quite similar. In general, uptake in samples from the border and infarcted regions was highest in the endocardium and lowest in the epicardium. These results demonstrate that preferential uptake of ^{18}F-labeled antimyosin MAb fragments in infarcted myocardium can be achieved as early as 2 hr after injection.

In the PET scans performed on Dogs 2-5, there was a suggestion of increased accumulation of ^{18}F activity in regions of the myocardium expected to be at risk in this model. Delineation of areas of infarcted tissue was complicated by the presence of high levels of ^{18}F activity in the blood pool. In the last dog studied, [^{13}N]ammonia perfusion and reperfusion images were acquired prior to injection of ^{18}F-labeled antimyosin Fab to better define regions with compromised perfusion. Using the [^{13}N]ammonia perfusion and reperfusion images, regions of interest were set. Regions 9, 10, and 11 had relatively high uptake and were considered to represent normal myocardium; and regions 3, 4, and 5 had the lowest activity levels and were considered to contain infarcted tissue. In Figure 2, the uptake of ^{18}F-labeled Fab in these regions of interest

Table 1. Tissue Distribution Data for ^{18}F-labeled Antimyosin MAb Fragments

Animal Number*	Slice Number	%ID/g Infarct	Infarct:normal Myocardium	Infarct: Blood
F(ab')$_2$ Fragment				
1	A	2.94×10^{-2}	16.0	2.2
	B	3.85×10^{-2}	20.6	2.9
2	A	2.42×10^{-2}	12.1	2.8
	B	2.73×10^{-2}	12.6	3.2
Fab Fragment				
3	A	4.80×10^{-2}	11.9	1.5
	B	5.00×10^{-2}	11.3	1.5
4	A	3.89×10^{-2}	10.9	1.3
	B	3.27×10^{-2}	8.7	1.1
5	A	3.76×10^{-2}	5.3	1.0
	B	6.06×10^{-2}	8.4	1.6

*With the exception of Dog 5, which was sacrificed at 3.5 post injection, these data were obtained at 2 hr.

FIGURE 1

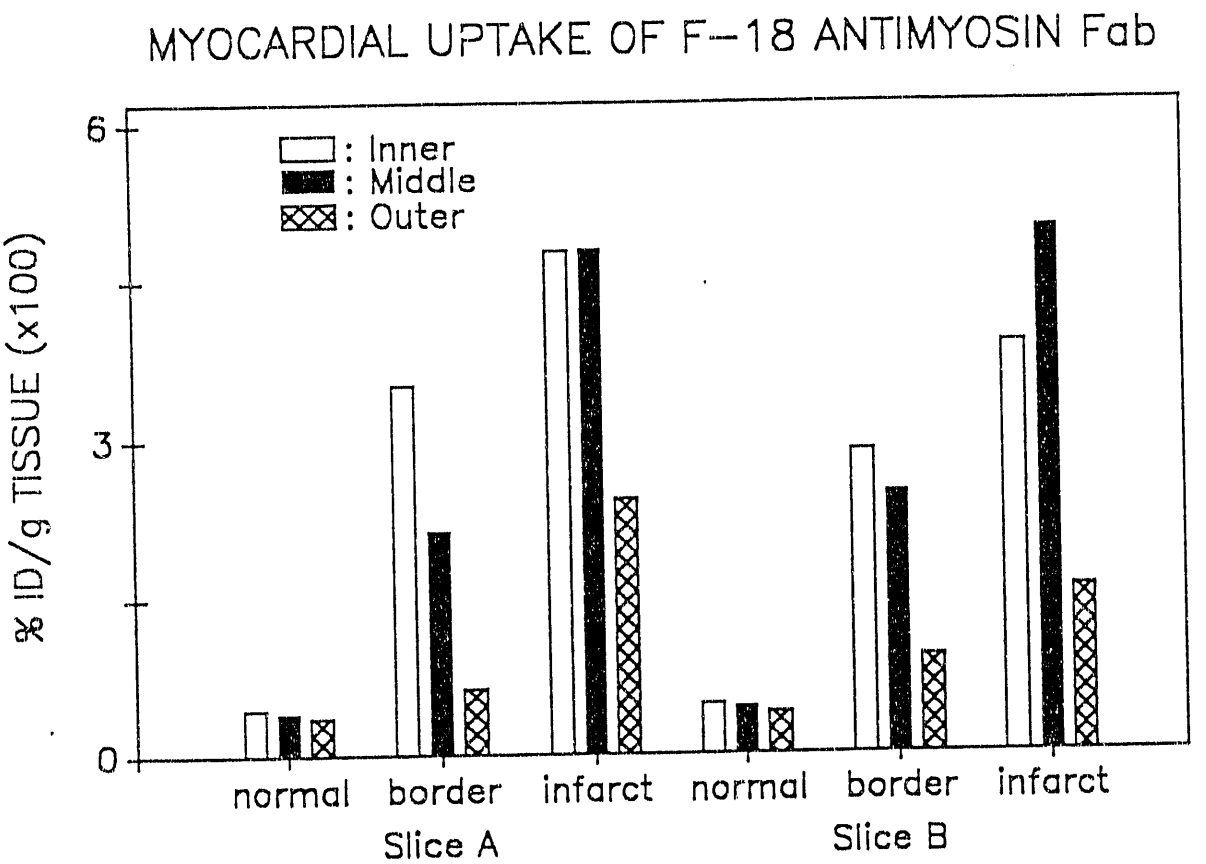
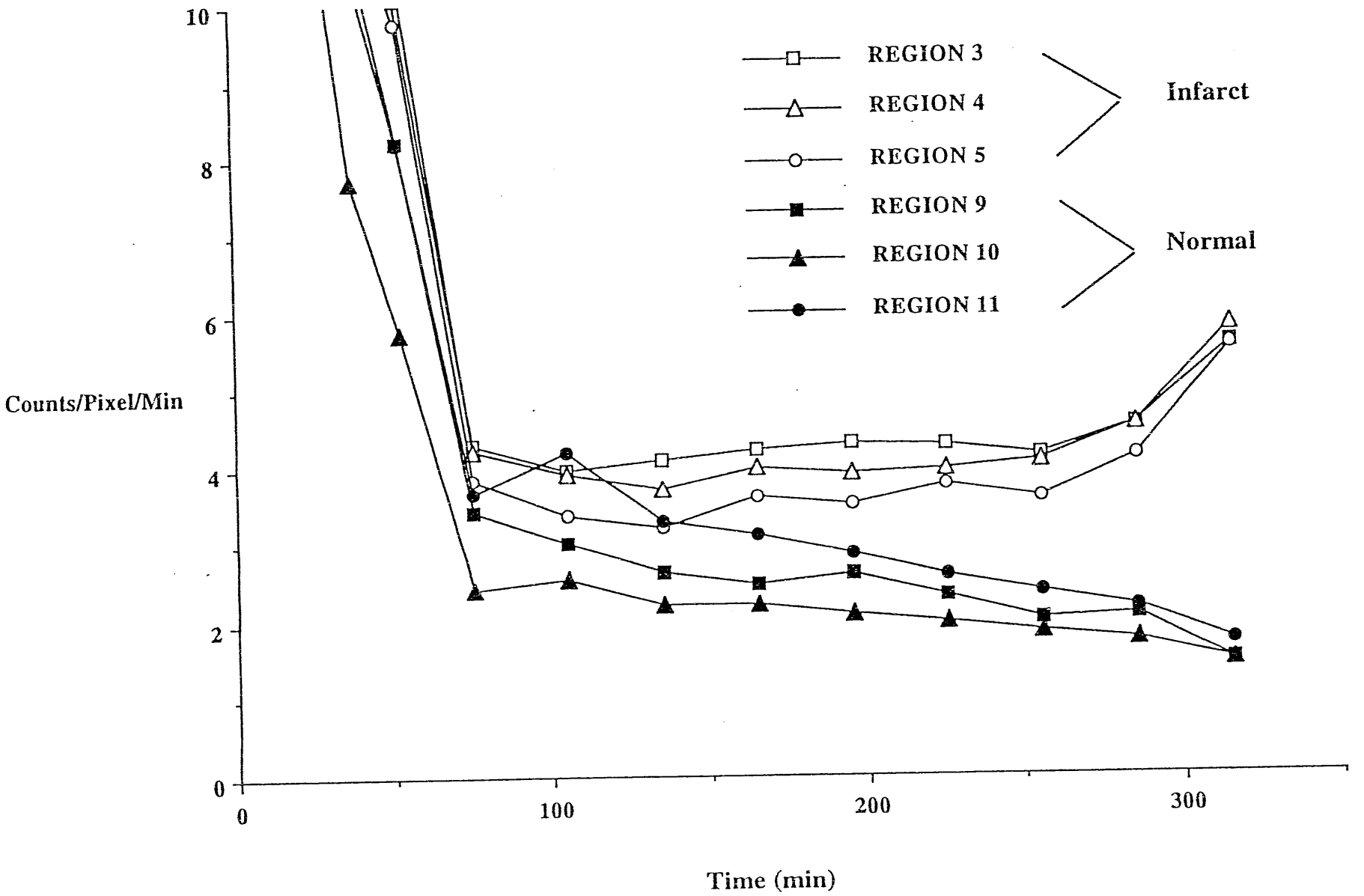

MYOCARDIAL UPTAKE OF F-18 ANTIMYOSIN Fab
: Inner
: Middle
: Outer
% ID/g TISSUE (x100)
6
3
0
normal
border
infarct
normal
border
infarct
Slice A
Slice B

FIGURE 2


Uptake of F-18 Antimyosin Fab
in Canine Myocardium
REGION 3
REGION 4
REGION 5
REGION 9
REGION 10
REGION 11
Infarct
Normal
Counts/Pixel/Min
Time (min)
0
2
4
6
8
10
0
100
200
300

is plotted as a function of time. Infarct:normal myocardium uptake ratios calculated from these images increased from 1.5:1 at 1 hr to 4.0:1 at 4 hr. These preliminary results suggest that ^{18}F-labeled antimyosin MAb fragments may be useful for imaging damaged myocardium; however, some form of blood pool subtraction probably will be required.

2. Labeling MAbs and Fragments with ^{18}F Using [^{18}F]SFB.

If PET data are to be useful for predicting dosimetry for radioimmunotherapy, the tissue distribution of the ^{18}F-labeled MAb and its catabolites must mimic those of the MAb when labeled with the therapeutic nuclide. Because of the promising results that we and others have obtained with [^{131}I]iodobenzoyl and [^{211}At]astatobenzoyl MAb conjugates, we were interested in developing a strategy that would result in the coupling of [^{18}F]fluorobenzoyl groups to a MAb. With ^{131}I and ^{211}At, synthesis of the labeled acylation agent was accomplished by electrophilic destannylation; however, this approach is not practical for ^{18}F since the specific activity of electrophilic fluorinating agents derived from [^{18}F]F_2 is too low.

Although most of our previous studies with ^{131}I and ^{211}At have utilized 3-halobenzoate esters, a *para* substituted compound was chosen for ^{18}F because nucleophilic fluorination with the activating group in the *meta* position is problematic. The method developed for the synthesis of N-succinimidyl 4-[^{18}F]fluorobenbenzoate ([^{18}F]SFB) is illustrated in Figure 3. This procedure involved three steps; [^{18}F]fluoride for trimethylammonium substitution on 4-formyl-N, N, N-trimethylanilinium triflate, oxidation to 4-[^{18}F]fluorobenzoic acid, followed by reaction with N-hydroxysuccinimide and dicyclohexylcarbodiimide to yield [^{18}F]SFB.

Since the carbodiimide-mediated preparation of N-succinimidyl esters normally is slow relative to the half life of ^{18}F, the yield for this reaction as a function of time was investigated. As shown in Figure 4, the radiochemical yield of [^{18}F]SFB increased from 32.0 ± 1.4% at 10 min to 87.9 ± 1.4% at 60 min. Since the amount of product available at the end of the reaction (effective yield) was essentially identical from 30-60 min, a 35-min esterification reaction was used in subsequent experiments. The overall radiochemical yield for the preparation of [^{18}F]SFB was about 25%. About 13 mCi of ^{18}F labeled ester could be prepared from 100 mCi of [^{18}F]fluoride in a synthesis time of 100 min. The conditions for labeling MAbs with [^{18}F]SFB were essentially identical to those originally developed for use with the N-succinimidyl ^{131}I and ^{211}At labeled esters. At MAb concentrations of about 3 mg/mL in pH 8.5 borate buffer, coupling efficiencies of 40-60% have been obtained after a 15-20 min reaction.

A preliminary evaluation of the immunoreactivity of ^{18}F-labeled Mel-14 F(ab')$_2$ was performed using a paired-label, single-point specific binding assay. The mean specific binding for the ^{18}F-labeled fragment was 58.1 + 2.5 %, a value slightly lower than that observed for Mel-14 F(ab)$_2$ labeled using S[^{125}I]IB (62.1 + 2.3 %; $P < 0.01$) and incubated with the same homogenates. These results are nearly identical to those reported previously for [^{131}I]iodobenzoyl and [^{211}At]astatobenzoyl conjugates of Mel-14

$F(ab')_2$ and considerably higher than those obtained when this fragment was radioiodinated using a conventional radioiodination method. Thus, the levels of cold material present in the ^{18}F-labeled ester preparation are low enough to avoid alteration of MAb immunoreactivity. It should be noted that the effect of other lower specific activity ^{18}F-labeled acylation agents on MAb immunoreactivity has not been determined.

In order for an ^{18}F-labeled MAb fragment to be useful for dosimetry studies, the pharmacokinetics of the ^{18}F label should approximate those of the nuclide used for radioimmunotherapy. Because of the promising results which have been obtained labeling MAbs using N-succinimidyl [^{131}I and ^{125}I]iodobenzoates, we anticipate initiating therapy trials using this method in the near future. For this reason, paired-label tissue distribution studies were performed in normal mice to compare the pharmacokinetics of Mel-14 $F(ab')_2$ labeled by reaction with the N-succinimidyl esters of both 4-[^{18}F]fluorobenzoate and 4-[^{125}I]iodobenzoate.

As shown in Figure 5, the tissue distribution of the two labels is quite similar over a period equivalent to over three half lives of ^{18}F. No significant differences between ^{18}F and ^{125}I uptake were observed in the spleen, blood, lung and muscle at any time point. In general, agreement in the tissue uptake of the two nuclides was better than that described in a previous report using a different ^{18}F-labeling method. Small but significant differences were seen in the liver at 2 h (^{18}F, 5.4 + 0.7 %ID/g; ^{125}I, 6.0 + 0.8 %ID/g; $P < 0.05$) and at 6 h (^{18}F, 4.6 + 0.5 %ID/g; ^{125}I, 5.5 + 0.6 %ID/g; $P < 0.01$). The most significant difference in the distribution of the two nuclides was noted in the kidneys where 11-46% lower levels of ^{18}F were observed at 0.5-6 h. The fact that this behavior may be related to differing excretion rates is suggested by the observation of complimentary differences in bladder activity (^{18}F, 4.6 + 2.8 %ID; ^{125}I, 1.9 + 1.2 %ID at 1 h). In contrast, 19-56% higher kidney and similar lower bladder activity of ^{18}F was seen when the biodistribution of antimyosin $F(ab')_2$ labeled using [^{18}F]SFBS and 3-[^{125}I]iodobenzoate esters were compared. It appears that the rate of urinary excretion of the $F(ab')_2$ fragment (or its labeled catabolites) decreases as the lipophilicity of the labeled acylation agent is increased; however, more comprehensive studies using the same MAb fragment would be required to confirm this speculation.

In summary, N-succinimidyl 4-[^{18}F]fluorobenzoate was synthesized at a no-carrier-added level and could be used to label a MAb $F(ab')_2$ fragment. Results from *in vitro* binding measurements and tissue distribution studies in normal mice suggest that this approach may be useful for labeling MAbs and other proteins with ^{18}F. Experiments are in progress to evaluate the utility of this ^{18}F-labeling method in a human tumor xenograft mouse model.

3. Development of a canine osteosarcoma cell line.

The goal of this effort is to develop a canine osteosarcoma line for use in *in vitro* binding assays and to create a canine osteosarcoma suitable for establishing xenografts in athymic mice. Samples are obtained from dogs undergoing amputations or at

FIGURE 3

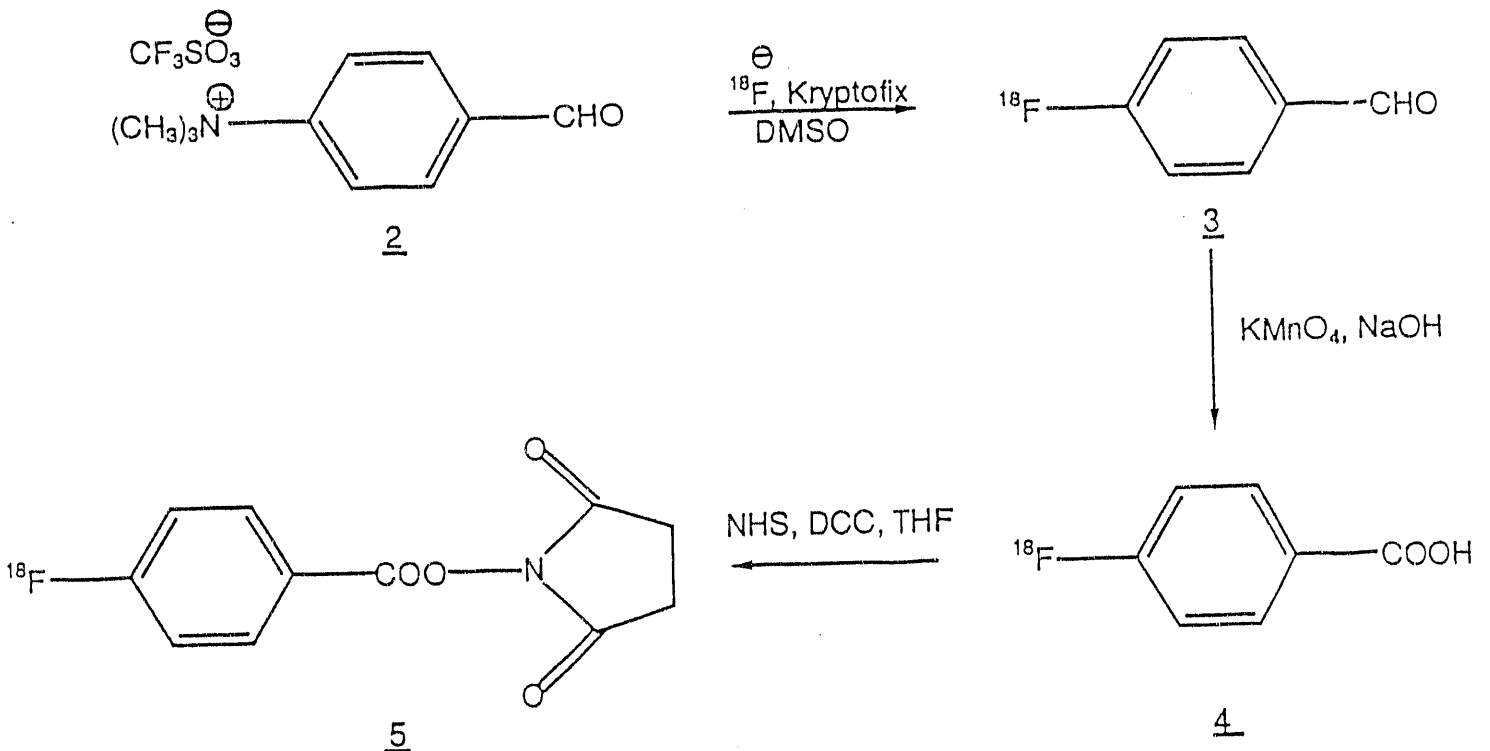

CF3SO3
(CH3)3N
CHO
2
18F, Kryptofix
DMSO
18F
CHO
3
KMnO4, NaOH
18F
COOH
4
NHS, DCC, THF
18F
COO
N
5

FIGURE 4

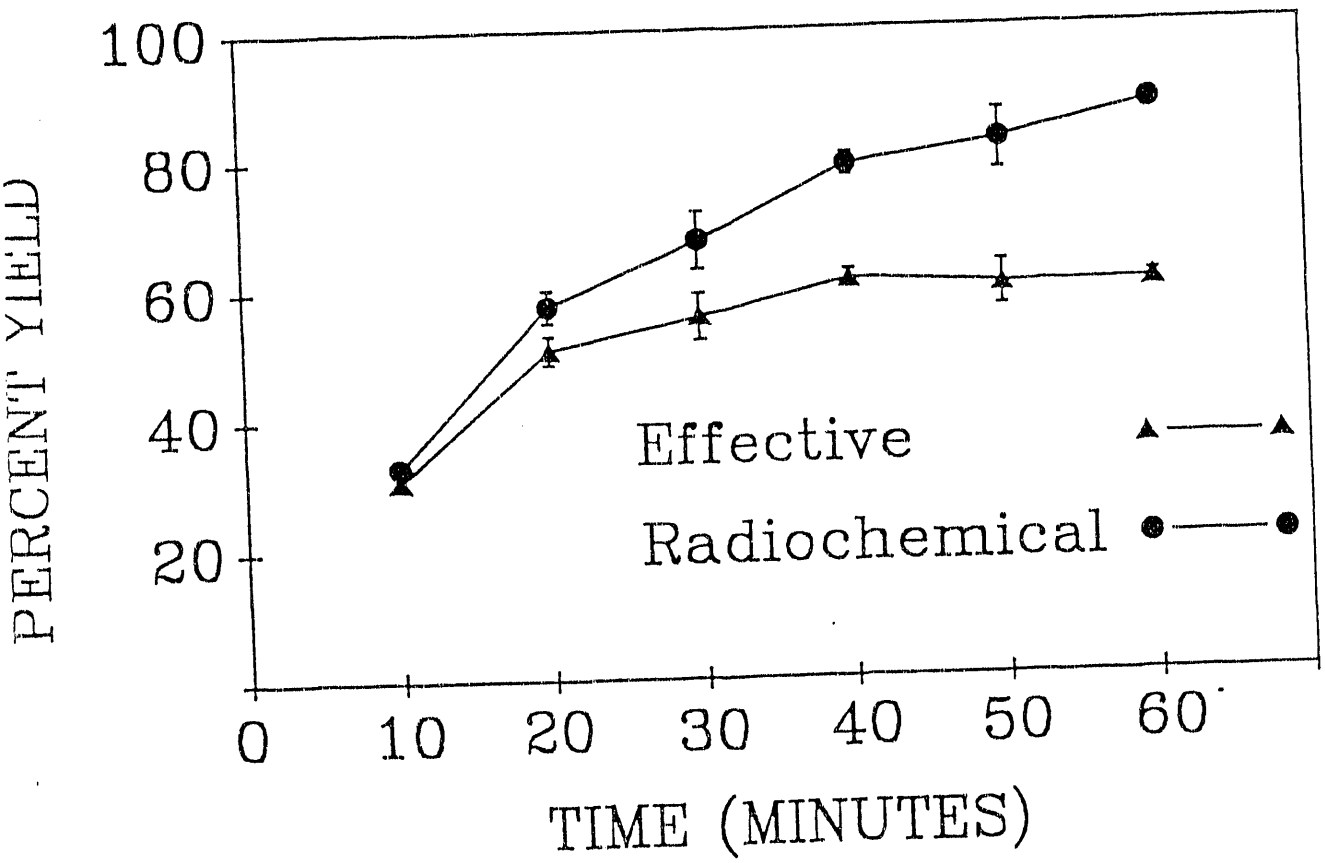
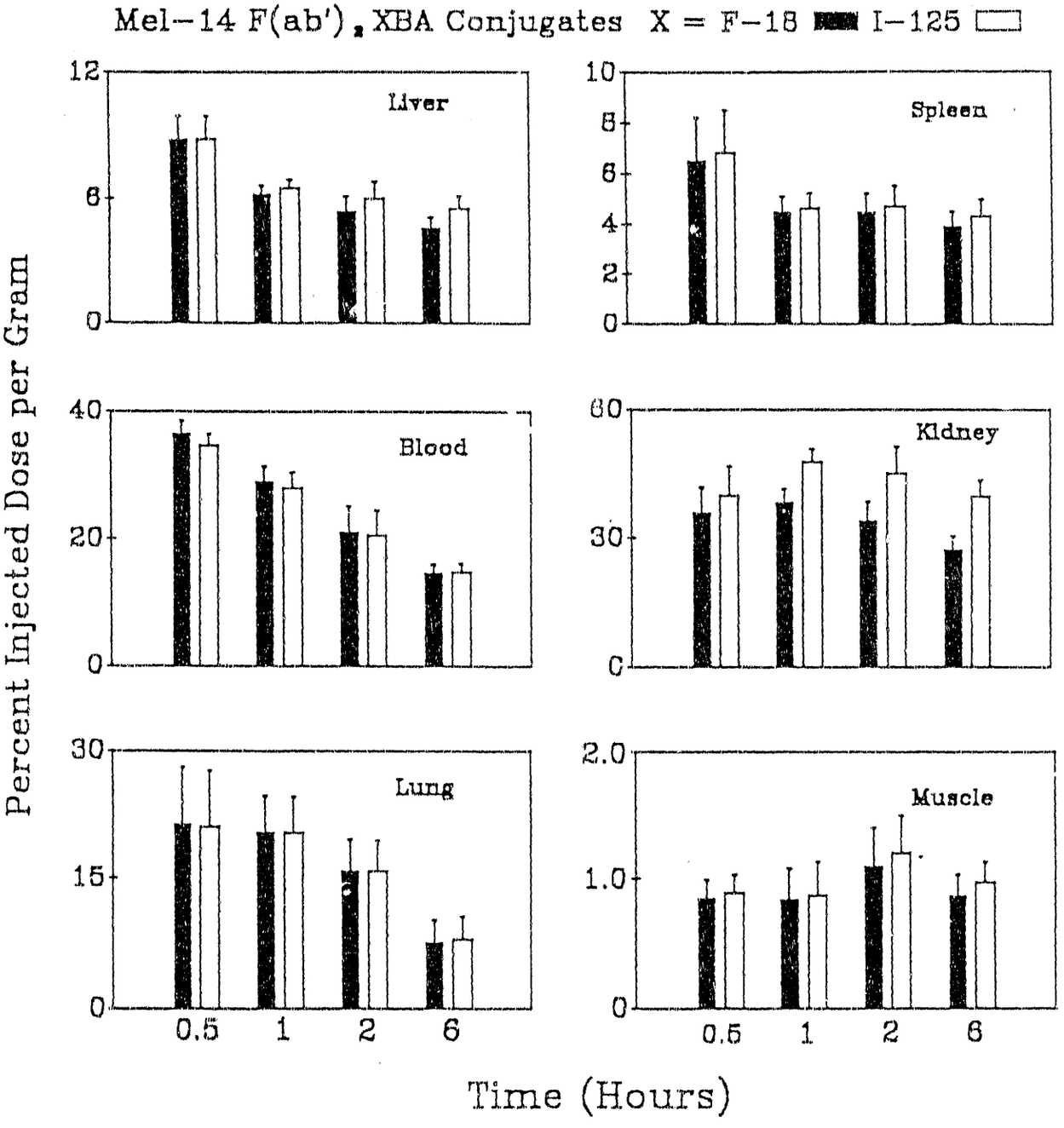

100
80
60
40
20
PERCENT YIELD
Effective
Radiochemical
0
10
20
30
40
50
60
TIME (MINUTES)

FIGURE 5


Mel−14 F(ab')2 XBA Conjugates X = F−18 I−125
Liver
Spleen
Blood
Kidney
Lung
Muscle
Percent Injected Dose per Gram
Time (Hours)
0.5
1
2
6

necropsy as a result having disseminated osteosarcoma. Surgical samples are preferred. Tissue is obtained as rapidly as possible. Tissue is isolated using aseptic techinques. Biopsy core samples and/or exposed tissues are placed in a small volume of pronase - collegenase solution. Once samples are obtained, all further work is done in a hood using sterile technique. If tissue obtained is contaminated with blood, the tissue is first rinsed in PBS. Following collection of all material, the tissue is minced into a fine brei then placed into a digestion flask. More digestion cocktail is added to a final volume of 50-100x the volume of tumor tissue isolated. The tissue is incubated in the digestion cocktail 15-30 min. Longer times are indicated if the cocktail does not appear to be increasing in turbidity. Following digestion, the cells are filtered through gauze into centrifuge tubes. Cells are spun at 2500 rpm for 10 min at 5°C. The supernatant is gently poured off and the pellets are resuspended in complete media. (RPMI 1640 is the base media used. Different additives will be used to optimize cell growth, i.e. insulin, 2BME, dexamethasone). Cell viability is determined and cells are seeded into flasks at high density. If there are cells attached the following day, the original media is transferred to new flasks and fresh media is placed on the attached cells in the original flasks. Once monolayers become confluent they are subcultured. Cells growing in suspension are propagated. Following initial growth cell type identification is done.

C. GRADUATE STUDENTS

No graduate school program exists in the Department of Radiology at Duke University. Two graduate students in the Department of Pathology, James Schuster and Joseph Ventigmalia, are working on Ph.D. projects funded in part by this grant. In addition, Michael Noska, a Department of Energy Occupational Health Physics Fellow in the graduate program at the University of North Carolina, Chapel Hill, is doing his Masters thesis on work performed at Duke in the laboratory of the principal investigator.

D. PUBLICATIONS SINCE LAST PROGRESS REPORT

1. Zalutsky, M.R., Garg, P.K., Vaidyanathan, G. and Garg, S. (1991) Methods for the radiohalogenation of antibodies. In: Applications for Enzyme Biotechnology, Plenum Press. Kelly, J.W. and Baldwin, T.O., eds. Plenum Press, New York; p 15-28.

2. Garg, P.K., Garg, S., DeGraff, W.G., Zalutsky, M.R. and Mitchell, J.B. (1992) 4-Fluorobenzylamine and phenylalanine methyl ester conjugates of 2-nitroimidazole: evaluation as hypoxic cell radiosensitizers. Int. J. Radiat. Oncol. Biol. Phys. 22:593-596.

3. Vaidyanathan, G. and Zalutsky, M.R. (1992) Labeling proteins with fluorine-18 using N-succinimidyl 4-[^{18}F]fluorobenzoate. Nucl. Med. Biol. 19:275-282.

4. Zalutsky, M.R., Garg, P.K., Johnson, S.H. and Coleman, R.E. (1992) Fluorine-18 antimyosin monoclonal antibody fragments: Preliminary investigation in a canine myocardial infarct model. J. Nucl. Med. 33:575-580.

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