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PRINCIPAL INVESTIGATOR: Raymond M. Reilly, Ph.D.

CONTRACTING ORGANIZATION: Toronto General Hospital
Toronto, Ontario M5G 2C4 Canada

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6. AUTHOR(S):
Raymond M. Reilly, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Toronto General Hospital
Toronto, Ontario M5G 2C4 Canada

E-Mail: raymond.reilly@utoronto.ca

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Ductal carcinoma in situ (DCIS) is an early stage of breast cancer treated by surgery and local radiotherapy. The surgeon has difficulty however in accurately defining the disease margins which may lead to re-excision or recurrent DCIS. Our objective is to develop novel radiopharmaceuticals for radioguided surgery (RGS) of DCIS to improve its surgical management. In the 2nd year, monoclonal antibodies (mAbs) CC49 and trastuzumab (Herceptin®) were tested for reactivity with DCIS specimens which demonstrated 60% and 20% reactivity respectively. We also completed preclinical testing of trastuzumab Fab labeled with indium-111 in mice implanted with human breast cancer xenografts which showed high and specific radiopharmaceutical tumor accumulation achieving tumor/blood ratios of >25:1 at 72 hours p.i. A gamma detecting surgical guidance system (C-Trak) was purchased and evaluated for sensitivity and spatial resolution using a framework to move the probe accurately in the x, y and z directions over phantom models of simulated DCIS lesions. Finally, an expression vector was constructed to produce a novel recombinant CC49 Fab fragment containing an integrated radiometal binding site for technetium-99m. Studies are continuing in the 3rd year and a Phase I trial is planned for the 4th year of the project.

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INTRODUCTION

Ductal carcinoma *in situ* (DCIS) is an early non-invasive stage of breast cancer in which malignant cells remain confined by the basement membrane of the ducts (1). The incidence of DCIS has been increasing due to widespread mammographic screening to detect breast cancer at an early stage and DCIS now represents 15-30% of all new cases of breast cancer diagnosed mammographically (2,3). DCIS is treated in most cases by breast conservation surgery (BCS) followed by local radiotherapy, but re-excision is frequently required due to the difficulty in determining "clear" margins at operation. In addition, residual disease may be present in up to 25% of patients assumed to be treated successfully, resulting in recurrent disease, which in about half of cases presents as invasive breast cancer (4,5). There is a need to improve the surgical treatment of DCIS through more accurate delineation of the extent of disease in order to decrease re-excision rates with their attendant costs and patient morbidity, and also decrease the rate of recurrence. Radioguided surgery (RGS) is a technique which employs highly specific radiopharmaceuticals directed towards malignant cells administered to the patient prior to surgery (6). The surgeon uses a sensitive γ-detecting probe intraoperatively to more clearly define the margins for resection of disease through identifying cancer cells specifically targeted by the radiopharmaceuticals. Our objectives in this research project are: 1) to develop novel monoclonal antibody Fab' fragments labeled with ^{111}In or ^{99m}Tc as radiopharmaceuticals for radioguided surgery of DCIS and evaluate these preclinically in tumor xenograft models, 2) project the sensitivity and resolution of the approach using phantom models of DCIS containing breast cancer cells targeted *in vitro* with the radiopharmaceuticals and 3) design and conduct a pilot Phase I clinical trial of radioguided surgery in a limited group of patients with DCIS.

BRIEF SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 1 (2000-2001)

The following is a brief summary of the research accomplished in the 1st year of the project. Please refer to the *August 2000 Annual Report* for a more detailed description.

Task 1: Identification of monoclonal antibodies reactive with DCIS by immunohistochemistry

The tumor-associated glycoprotein-72 (TAG-72) cell surface mucin is reported to be expressed on >80% of DCIS (7) and the HER-2/neu transmembrane receptor tyrosine kinase has been detected in 60-80% of DCIS (8). Monoclonal antibodies (mAbs) CC49 or trastuzumab (Herceptin®) which react with TAG-72 or HER-2/neu respectively were evaluated for immunoreactivity with tissue sections of benign breast disease, pure DCIS, invasive ductal carcinoma (IDC) or combined DCIS and IDC. These studies showed that mAb CC49 reacted with 3/4 (75%) DCIS specimens, 9/20 (45%) IDC specimens and 4/14 (28%) of combined DCIS and IDC. Only very slight staining of benign breast disease was observed for mAb CC49 in 4/6 (66%) cases. Trastuzumab (Herceptin®) reacted with 2/4 (50%) DCIS specimens and 2/10 (20%) cases of IDC. There was no reactivity of trastuzumab (Herceptin®) with benign breast disease. The number of cases of DCIS, IDC, benign breast disease stained with these two antibodies has been expanded in Year 2 and these results are described later in this report.

***Task 2*: Construct novel radiopharmaceuticals for radioguided surgery of DCIS**

Radiopharmaceuticals consisting of mAb Fab' fragments of trastuzumab (Herceptin®) or anti-TAG72 mAb CC49 labeled with ^{111}In or ^{99m}Tc were constructed and evaluated *in vitro* for their purity, homogeneity and retention of immunoreactivity. Fab' fragments offer the advantages of rapid tumor localization and clearance from the blood and normal tissues as well as low immunogenicity in humans (9).

Preparation of Fab' fragments of trastuzumab (Herceptin®) and radiolabeling with ^{99m}Tc, ^{111}In or ^{125}I. Trastuzumab (Herceptin®) Fab' fragments were prepared by enzymatic digestion of intact IgG using immobilized papain and were purified on a Protein-A column. SDS-PAGE and Western blot analysis of the Fab' fragments showed a purity >90%. Fab' fragments of trastuzumab (Herceptin®) were labeled with ^{99m}Tc as described by Ultee et al. (10). The antibody fragments were conjugated with hydrazinenicotinamide (HYNIC) for subsequent reaction with ^{99m}Tc-glucoheptonate. Depending on the molar ratio of HYNIC:protein used in the reaction (5:1 to 20:1), a substitution level of 1.2-11.6 HYNIC groups/molecule Fab' was obtained. The radiochemical purity of ^{99m}Tc-HYNIC-trastuzumab Fab' was >94% measured by instant thin layer chromatography (ITLC-SG). Fab' fragments of trastuzumab (Herceptin®) were labeled with ^{111}In by derivatization of the proteins with diethylenetriaminepentaacetic acid (DTPA) (12). Fab' fragments were derivatized with DTPA at a 10:1 molar ratio, purified by size-exclusion chromatography and labeled with ^{111}In acetate. The radiochemical purity of ^{111}In-DTPA-Fab' was >90% measured by ITLC-SG. Fab' fragments of trastuzumab (Herceptin®) and intact IgG were labeled with ^{125}I using the Iodogen™ method (12) and purified by size-exclusion chromatography. The radiochemical purity of ^{125}I-trastuzumab (Herceptin®) Fab' and intact IgG was >99% measured by paper chromatography developed in 85% methanol/water.

The immunoreactivity of trastuzumab (Herceptin®) Fab' fragments was assessed by competition binding assays against HER-2/neu (+) SKBr-3 breast cancer cells. The K_a-values were: 2.7×10^8 and 6.6×10^7 L/mol for non-derivatized IgG and Fab' respectively and 5.9×10^7 and 1.5×10^7 L/mol respectively for HYNIC-derivatized IgG and Fab'. These assays demonstrated a 4-fold decrease in binding affinity for trastuzumab Fab' compared to intact IgG and a further 4-fold decrease in affinity following derivatization with HYNIC for labeling with ^{99m}Tc. The immunoreactivity of trastuzumab (Herceptin®) IgG or Fab' fragments labeled with ^{125}I was evaluated in a direct radioligand binding assay using HER-2/neu (+) SKBr-3 breast cancer cells. The binding affinity of ^{125}I-labeled trastuzumab Fab' was decreased 2-fold compared to the intact IgG (K_a 7.7×10^7 vs. 1.4×10^8 L/mol respectively).

Preparation of Fab' fragments of monoclonal antibody CC49. The TAG-72 monoclonal antibody CC49 was purified from mouse ascites by affinity chromatography on a Protein G column (Pierce). Fab' fragments of CC49 were prepared by enzymatic digestion of the intact IgG using immobilized papain and purified as described for trastuzumab (Herceptin®) Fab'. The purity of the Fab' fragments was determined by SDS-PAGE, Western blot and size-exclusion HPLC. CC49 Fab' migrated as a single band on SDS-PAGE with the expected M_r of 50 kDa which was positive by Western blot using an anti-mouse Fab antibody. Size-exclusion HPLC further demonstrated that the purity of the Fab' fragments was >92%.

The immunoreactivity of mAb CC49 IgG and Fab' fragments were assessed by a solid phase competition binding assay using bovine submaxillary mucin (BSM) as a source of the TAG-72 antigen and ^{125}I-labeled CC49 IgG as a radiotracer. These assays showed that there was more than a 100-fold decrease in binding affinity of CC49 Fab' compared to intact CC49 IgG (K_a 1.1 $\times$ 10^6 vs. 1.2 $\times$ 10^8 L/mol respectively). Alternative methods of preparing Fab' fragments of mAb CC49 will be required to preserve the immunoreactivity. We are now exploring the possibility of constructing a recombinant Fab' fragment of mAb CC49 containing an integrated radiometal binding site to address these issues (described later in this report).

Task 3: Conduct preclinical testing of radiopharmaceuticals in animal tumor xenograft models

Tumor imaging and biodistribution studies of trastuzumab (Herceptin®) IgG and Fab'. The tumor and normal tissue localization of trastuzumab (Herceptin®) labeled with ^{99m}Tc or ^{111}In was evaluated in athymic mice implanted s.c. with HER-2/neu positive BT-474 human breast cancer xenografts. The tumor and normal tissue uptake was expressed as percent injected dose/g (% i.d./g) and as tumor/normal tissue (T/NT) ratios and compared with that for Fab' fragments of a control non-specific mAb (anti-CD33 HuM195). These studies demonstrated high tumor accumulation of ^{99m}Tc-trastuzumab (Herceptin®) Fab' (10.7 ± 3.5 % i.d./g) at 24 h post-injection which was significantly greater (t-test, $p<0.05$) than that for control HuM195 Fab' (2.6 ± 0.5 % i.d./g) demonstrating that the tumor uptake was specific for the HER-2/neu receptor. The major normal tissue uptake of ^{99m}Tc-trastuzumab (Herceptin®) Fab' was in the kidneys with smaller amounts deposited in the liver, lungs and spleen. The T/NT ratios at 24 h for ^{99m}Tc-trastuzumab (Herceptin®) Fab' were significantly higher (t-test, $p<0.05$) than those for HuM195 Fab' again demonstrating specific localization in BT-474 tumors. The tumor/blood ratio for ^{99m}Tc-trastuzumab (Herceptin®) Fab' at 24 h (3.2 ± 0.7) was significantly greater than that for HuM195 Fab' (0.9 ± 0.2). The short half-life of ^{99m}Tc (6 hours) however does not permit detection beyond 24 h post-injection. In order to determine if improvements in tumor localization may be achieved at later time points, trastuzumab (Herceptin®) was labeled with ^{111}In (half-life 67 hours). The T/NT ratios were increased for ^{111}In-trastuzumab Fab' compared to ^{99m}Tc-trastuzumab Fab'. For example the tumor/blood ratio for ^{111}In-trastuzumab Fab' was 4.1 ± 0.6 and 10.3 ± 1.0 at 24 h and 48 h post-injection respectively compared to 3.2 ± 0.7 for ^{99m}Tc-trastuzumab Fab' at 24 h post-injection. These results suggested that ^{111}In-labeled trastuzumab (Herceptin®) Fab' may be advantageous for radioguided surgery of DCIS since it permits detection at time points when the T/NT ratios are highest. Nevertheless, BT-474 tumors were visualized by γ-camera imaging with either ^{99m}Tc- or ^{111}In-trastuzumab(Herceptin®) Fab'.

Tumor imaging and biodistribution studies of monoclonal antibody CC49 IgG and Fab'. Tumor imaging and biodistribution studies were performed with mAb CC49 Fab' labeled with ^{99m}Tc in athymic mice bearing s.c. TAG-72 (+) LS174T human colon cancer xenografts. The LS174T xenograft model is not a breast cancer model but nevertheless is a standard model for evaluating the targeting of radiolabeled TAG-72 mAbs to tumors *in vivo*. These studies demonstrated that ^{99m}Tc-labeled CC49 Fab' localized in TAG72 (+) LS174T tumor xenografts despite the significantly decreased immunoreactivity observed *in vitro* and was rapidly eliminated from the blood and normal tissues producing tumor/blood ratios of >4:1 at 24 h post-injection. The rapid tumor uptake and clearance from normal tissues allowed successful tumor

imaging as early as 2 h post-injection (not shown). Nevertheless, it is likely that significantly improved tumor targeting could be achieved by generating a CC49 Fab' fragment with preserved immunoreactivity and for this reason, we are exploring methods of generating these fragments by recombinant DNA techniques as previously described.

SUMMARY OF RESEARCH COMPLETED IN YEAR 2 (2001-2002)

Task 1: (Continued) Identification of Monoclonal Antibodies Reactive With DCIS by Immunostaining

The suitability of the antibodies for radioguided surgery of DCIS was screened by immunohistochemical staining of a panel of tissue sections including DCIS, invasive breast cancer, and benign breast disease. In Year 2, we significantly expanded the number of cases of breast surgical specimens to test their immunoreactivity with mAbs trastuzumab (Herceptin®) and CC49 by immunohistochemistry using the methods described in *August 2001 Annual Report*. The stained slides were examined by a pathologist (Dr. Harriette Kahn).

As shown in **Table 1**, trastuzumab was reactive with 19% of DCIS sections, but stained no cases of benign breast lesions and only 10% of invasive breast cancer. It has been previously shown in the literature that HER2/neu receptor is expressed in about 60% of DCIS by immunostaining using murine antibodies. The reason that trastuzumab had a lower reactive rate with DCIS than other mouse antibodies (~60% to 80%) (13,14) is unclear. The subtype and grade of DCIS reportedly plays an important role in the expression of HER2/neu receptor (13,14). HER2/neu receptor is positive in up to 77% of high-grade, comedo-type DCIS versus only about 15% (14) of non-comedo type DCIS. To further verify whether the low reactivity with DCIS by trastuzumab is due to the sample selection, we plan to stain more DCIS cases and subgroup them by subtype and grade.

Table 1. Reactivity of trastuzumab (Herceptin®) and CC49 with DCIS, invasive breast cancer and benign breast disease.

Pathology	n	Reactivity with Trastuzumab (Herceptin®)	Reactivity with CC49
Benign breast disease	6	0	4†
Pure DCIS	32	6	22
Invasive ductal carcinoma	20	2	9
Combined DCIS and invasive ductal carcinoma	14	1	4

† Minor staining of secretory surface epithelium

Interestingly, we have also compared the immunoreactivity of trastuzumab (Herceptin®) with DCIS specimens with that of the more commonly used CB11 antibody used for immunostaining for HER-2/neu expression or the HercepTest® (**Table 2**). These studies demonstrated that there was a much lower immunoreactivity of trastuzumab (Herceptin®) with DCIS than with the other techniques. The clinical significance of these findings is not clear at the present time but they do suggest that immunostaining of breast cancer specimens for HER-2/neu using CB11 or the HercepTest® may not predict reactivity with trastuzumab (Herceptin®) in every case.

Table 2. Comparison of immunoreactivity of trastuzumab (Herceptin®), CB11 and HercepTest® with DCIS specimens.

	Herceptin®	CB11	HercepTest®
Positive	6	11	11
Negative	26	17	13
Not tested	0	4	8
Total	32	32	32

The TAG-72 mAb CC49, on the other hand, was reactive with a much higher percentage (69%) of DCIS sections compared with trastuzumab (Herceptin®). It showed slight cross-reactivity however with benign breast lesions (**Table 1**). The reactivity with IDC and combined DCIS/IDC was also high for CC49. Overall, CC49 is strongly reactive with the majority of DCIS surgical specimens. This suggests a promising role of CC49 for radioguided surgery of DCIS.

Task 2*: *(Continued) Construct novel radiopharmaceuticals for radioguided surgery of DCIS.

As we reported in *August 2001 Annual Report*, Fab fragments of CC49 generated by enzymatic digestion of IgG using papain showed significantly reduced binding affinity with TAG-72 antigen in ELISA assays. Further decreases in affinity were observed when the Fab fragments were derivatized with hydrozinenicotineamide (HYNIC) required for labeling with ^{99m}Tc. The suboptimal properties of enzymatically-generated Fab fragments of CC49 strongly suggested therefore that an alternative method of preparing the Fab fragments of CC49 was required. We are now exploring a novel strategy to construct a recombinant CC49 Fab which contains an integrated metal-binding site (His_6) for direct labeling with ^{99m}Tc without the need to introduce HYNIC metal chelators. The following describes research conducted in 2001-2002 to construct, express and perform preliminary characterization of the novel CC49 recombinant Fab.

Cloning of L and Fd (V_H + C_H1) of CC49 from CC49 Hybridoma Cells. MAb CC49 is a murine $IgG_{1,\kappa}$. CC49 hybridoma cells were provided by Dr. J. Schlom in NCI (U.S.) through a Material Transfer Agreement with Dr. Reilly. Fab fragments are composed of the light chain (L), variable region of the heavy chain (V_H) and the C_H1 domain up to the first cysteine residue in the hinge region that forms the disulfide bond with the L chain. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to clone genes encoding L and Fd from the CC49 hybridoma cells. Briefly, the total RNA was extracted from 10^6 hybridoma cells using RNeasy kit (QIAGEN) then reverse-transcribed into single-stranded cDNA primed with random hexamer primers (Invitrogen). The target genes were amplified by PCR from cDNA using primers specific for the variable regions (15), C_κ region (16) and C_γ region (17,18) of CC49. The purified PCR products with correct DNA size corresponding to L and Fd were then directly cloned into TA cloning® pCR2.1 vector (Invitrogen) individually (pCR2.1-L and pCR2.1-H) and sequenced. The correct DNA sequence for the L and Fd genes were confirmed.

Vector Design for CC49 Fab Expression. The yeast expression system is used because it favors correct protein folding and the formation of disulfide bonds in the cytoplasm (19). The methylotrophic *Pichia pastoris* (Invitrogen) is used as the expression host. The secretion-vector pPICZαA, which contains an α-factor signal peptide directing secretion of the expressed

proteins into the culture medium, is used to construct expression vectors. The expression of the heterologous proteins is driven by a methanol-inducible alcohol-oxidase-promoter (5'*AOX I*) in pPICZαA. L and Fd are co-expressed and the folded Fab fragments are formed and secreted into the culture medium. A FLAG tag (DYKDDDDK) is fused to the N-terminus of L and a His_6 tag is fused to the C-terminus of Fd via a flexible GGGGS linker. The tags are designed for affinity purification of Fab. After purification, the FLAG tag will be cleaved by enterokinase and the His_6-tag will be maintained for subsequent labeling with ^{99m}Tc.

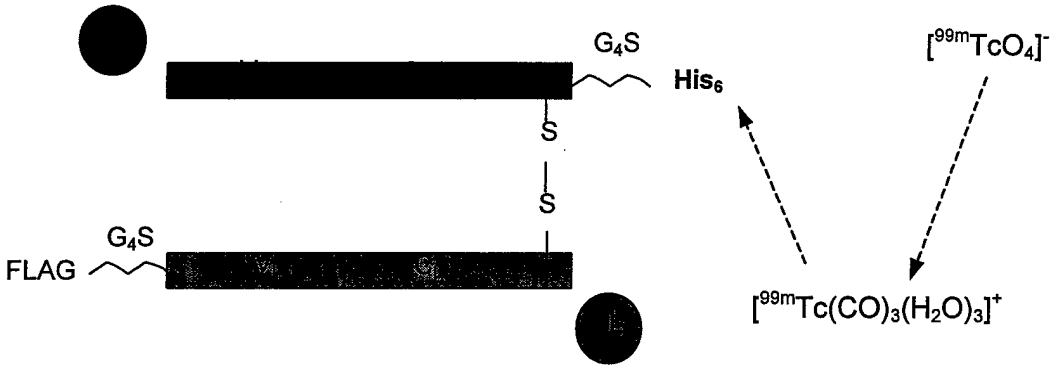
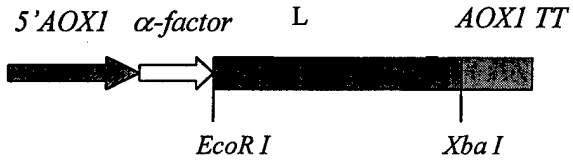


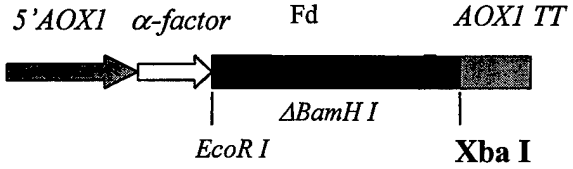
Fig. 1. Schematic representation of recombinant CC49 Fab construct.

Subcloning of L and Fd into pPICZαA Vector. L and Fd were amplified from pCR2.1-L and pCR2.1-H by PCR. The primers (L_FOR, L_REV, H_FOR and H-REV) incorporated restriction sites and the affinity tags. **L_FOR** (5'CTAGTGAATTC**GACTACAAGGACGACGACGACAAG** GGTGGCGGTGGCTCGGACATTGTGATGTCACAGTCTCCATC3') containing an *EcoR I* site (underlined) and FLAG sequence (bold) and **L_REV** (5'CACTGTCTAGATCACTAACACTCATTCCTGTTGAA GCTCTTGACAATGG3') containing an *Xba I* site (underlined) were used to amplify L from pCR2.1-L. **H_FOR** (5'CTAGTGAATTCCAGGTTCAGTTGCAGCAGTCTGACGCTGAGTTG3') containing an *EcoR I* site (underlined) and **H_REV** (5'CACTGTCTAGATCATCA**ATGATGATGATGATGATG**CGA GCCACCGCCACCACAATCCCTGGGCACAATTTTCTTGTC3') containing a His_6 tag (bold) and an *Xba I* site (underlined) were used to amplify Fd from pCR2.1-H. Two stop codons were placed at 3' *Xba I* site of **L_REV** and **H_REV**. The *BamH I* site within Fd was disrupted by site-directed mutagenesis. The amplified L and Fd genes flanked by *EcoR I* and *Xba I* sites were digested and ligated into a pPICZαA vector individually. The vectors were denoted as pPIC-**L** and pPIC-**Fd**.

pPIC-**L**



pPIC-**Fd**



Construction of Vectors for Co-expression of L and Fd. The expression-cassette in pPICZαA vector consists of the 5'*AOX I* promoter, the gene of interest, ie L or Fd, and the transcriptional terminator (TT). To co-express L and Fd, the L and Fd expression-cassettes, ie 5'*AOX1*-**L**-TT and 5'*AOX1*-**Fd**-TT, were placed in a single pPICZαA vector by using a similar strategy as described by Lange et al.(20). The L expression cassette (5'*AOX1*-**L**-TT) was amplified by PCR from pPIC-**L** with a disrupted *Pme I* site in 5'*AOX1* introduced by site-directed mutagenesis. Amplified 5'*AOX1-(ΔPmeI)***L**-TT was then cloned into a pPIC-**Fd** vector downstream of 5'*AOX1*-**Fd**-TT at the *BamH I* site to generate a co-expression vector pPIC-**Fd**-*(ΔPmeI)***L**. Similarly, a co-expression vector pPIC-**L**-*(ΔPmeI)***Fd** was constructed by placing 5'*AOX1-(ΔPmeI)***Fd**-TT downstream of the 5'*AOX1*-**L**-TT cassette in pPIC-**L** **(Fig. 2)**. The orientation of the expression cassettes was determined by PCR and the cloned genes were sequenced.

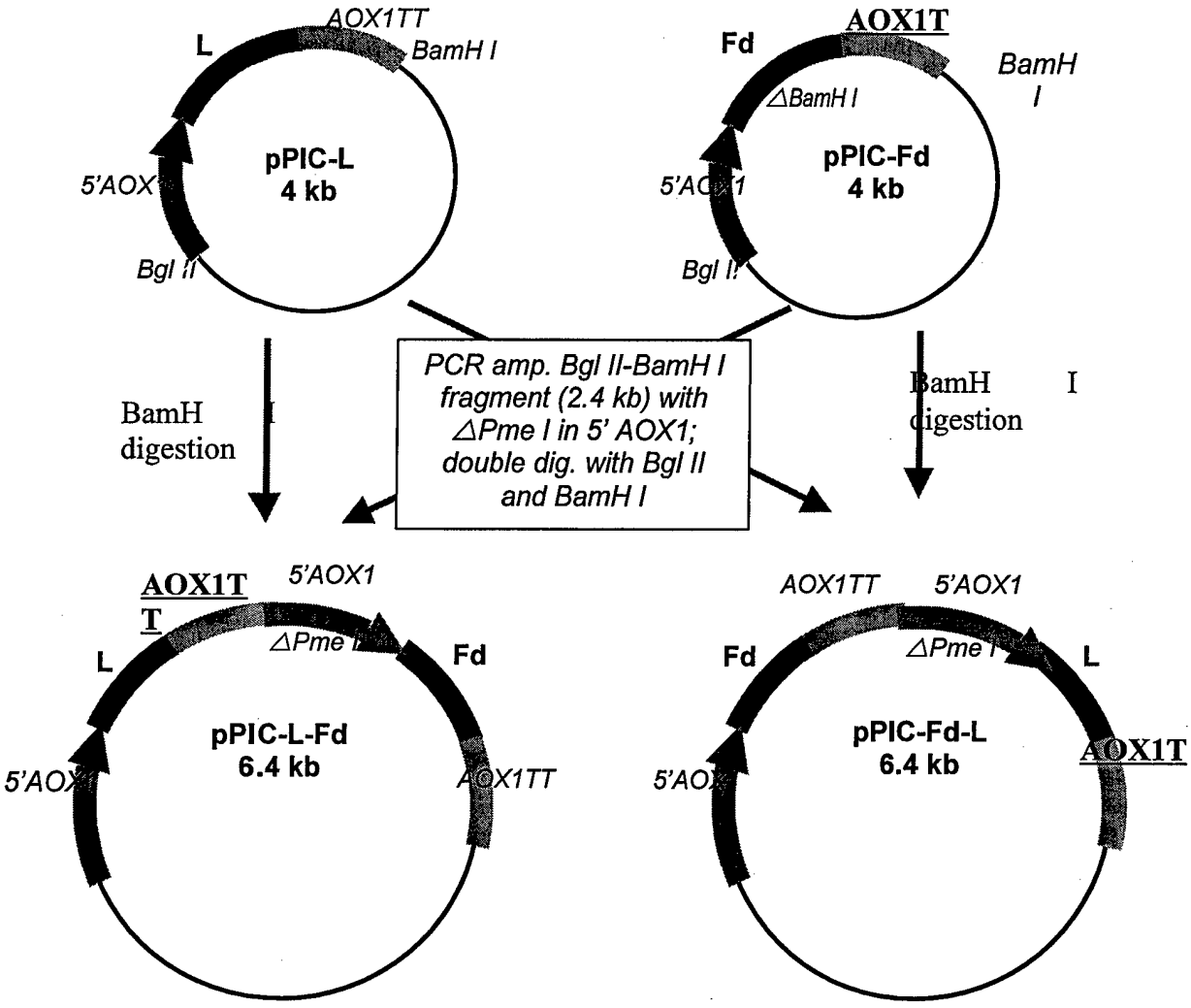


Fig. 2. Construction of co-expression vectors for recombinant CC49 Fab.

Expression of Recombinant CC49 Fab in Yeast (*Pichia pastoris*) and Purification. The pPIC-**L**-*(ΔPmeI)***Fd** and pPIC-**Fd**-*(ΔPmeI)***L** vectors were linearized at the unique *Pme I* site in

5'*AOX1* then electro-transformed into *Pichia pastoris* KM71H cells. The protein expression was induced with 0.5% methanol (v/v) added in the culture. The optimal expression time was determined by measuring protein expression by ELISA. Briefly, 2μg of bovine submaxillary mucin (BSM, Sigma), a source of the TAG-72 antigen, was coated onto wells in a 96-well microELISA plate at 4°C overnight. Non-specific binding sites were blocked by incubating the wells in 5% BAS in PBS for 1 h at 37 °C. 100 μl of the culture medium that was sampled at 24, 48, 72 and 96 hours after methanol induction were added to the wells. The plate was then incubated for 16 h at 4 °C. The wells were washed with PBS and the supernatant discarded. 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse Fab was then added to each well and incubated for 1 h at room temperature. The wells were washed again with PBS. 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) was added to the wells and incubated briefly allowing for color to develop. The reaction was stopped by adding 2 M H_2SO_4. The absorbance of the wells in the plate was read at 450 nm. The optimal expression was determined to be at 72 h after 0.5% methanol induction. **Table 3** shows the absorbance at 450 nm for clone #24-5 (pPIC-Fd-L) and clone #5-3 (pPIC-L-Fd) at 72 h after methanol induction. The sampled culture medium in the assay for #24-5 (pPIC-Fd-L) and clone #5-3 (pPIC-L-Fd) was concentrated 7 times using Centricon-30 micro-concentrators (Millipore).

Table 3. Absorbance at 450 nm (A_{450}) for clone #24-5 (pPIC-Fd-L) and clone #5-3 (pPIC-L-Fd) at 72 h after methanol induction. The fresh medium was used as a negative control and intact CC49 IgG was used as a positive control.

	1	2	3	4	5	6	7
	Blank	Clone Fd-L #24-5	Clone L-Fd #5-3	Control (-)	Control (-)	CC49 IgG 0.625 μg/ml	CC49 IgG 2.5 μg/ml
A	0.000	**0.168**	**0.215**	0.026	0.021	0.038	0.951
B	-0.000	**0.146**	**0.201**	0.021	0.023	0.035	0.968

A small amount of recombinant Fab (rFab) was purified by Ni-NTA affinity chromatography (QIAGEN) to confirm its identity and validate the purification method. SDS-PAGE (**Fig. 3**) showed one major band at ~53kDa (77%) and one minor band at ~27kDa (23%) under nonreducing conditions for rFab. The ~53kDa product dissociated into ~27kDa proteins under reducing conditions (with β-mercaptoethanol). Both bands (~53 kDa and ~27kDa) were reactive with goat anti-mouse Fab by Western blot (**Fig. 3**) indicating that the ~53kDa band corresponded to recombinant Fab. The purity of Fab was 77% by gel densitometry after this single step purification. The molecular weight of rFab is expected to be greater than the enzymatically generated Fab (eFab) due to inclusion of a His_6 and a FLAG tag. These two tags contribute 2.8 kDa. This explains the slight difference in molecular weight between rFab and eFab as shown on the SDS-PAGE gel and Western blot.

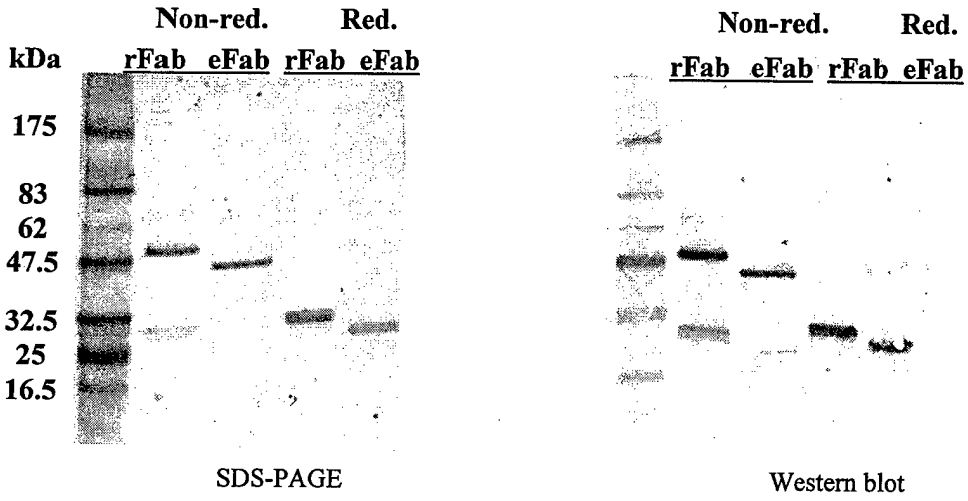


Fig. 3. SDS-PAGE and Western blot of recombinant CC49 Fab expressed in *Pichia pastoris*.

***Task 3:* (Completion) Preclinical Testing of Radiopharmaceuticals in Animal Tumor Xenograft Models**

Biodistribution Studies of ^{111}In-labeled Fab Fragments of Trastuzumab (Herceptin®). The short half-life of ^{99m}Tc (6 hours) does not permit sensitive detection beyond 24 h post-injection of ^{99m}Tc labeled radiopharmaceuticals. In order to determine if improvements in tumor localization may be achieved at later time points, the biodistribution of ^{111}In-labeled Fab fragments of trastuzumab were studied in a breast cancer mouse xenograft model. Fab fragments were generated by digestion using papain as previously described *(August 2001 Annual Report)*. Fab then was labeled with ^{111}In (half-life of 67 hours) by modification of the proteins with the metal chelator, diethylenetriaminepentaacetic acid (DTPA) using methodology previously described (9). Fab fragments (5 mg/mL in 50 mM sodium bicarbonate buffer pH 7.4) were reacted with a 10-fold molar excess of the bicyclic anhydride of DTPA (Sigma), then purified from excess-free DTPA by size-exclusion chromatography on a Sephadex G-25 mini-column. Purified DTPA-trastuzumab Fab was radiolabeled by incubation with ^{111}In acetate for 30 mins at room temperature. ^{111}In-acetate was prepared by mixing equal volumes of ^{111}In chloride (MDS-Nordion) with 1 M acetate buffer pH 6.0. The radiochemical purity of ^{111}In-trastuzumab Fab was >90% as measured by silica gel-instant thin layer chromatography (ITLC-SG) developed in 100 mM sodium citrate pH 5.0. The specific activity achieved was 1 μCi/μg. HER2/neu positive BT-474 human breast cancer xenografts were established in female nude mice as previously described *(August 2001 Annual Report)*. Approximately 30 μg of trastuzumab Fab labeled with ^{111}In was injected through tail vein into each mouse (30 μCi/mouse) for test groups. The control group of mice was injected with 30 μg of HuM195 (humanized anti-CD33 monoclonal antibody) Fab labeled with ^{111}In (30 μCi/mouse). Tumor uptake (percent injected dose per gram, % ID/g) and the tumor/normal tissue (T/NT) ratios at 24, 48 and 72 h after injection of ^{111}In- Fab are shown in **Fig. 4.**

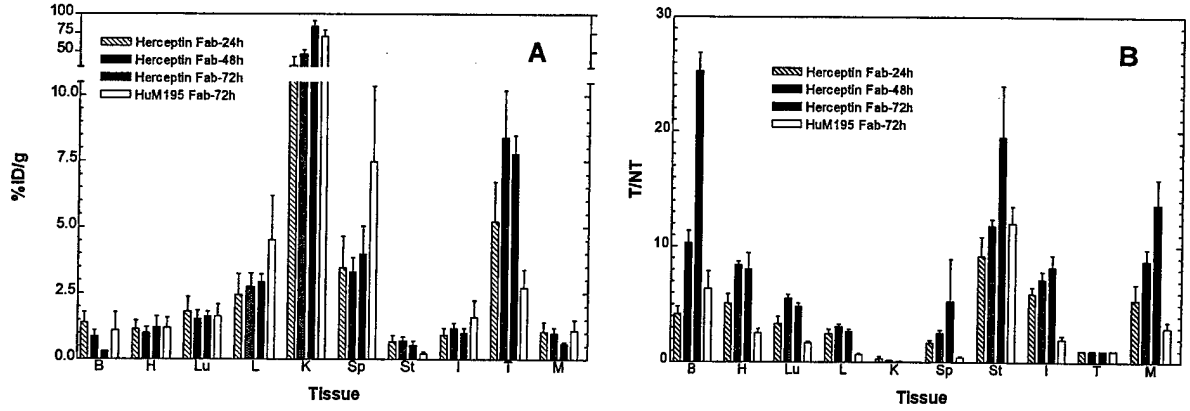


Fig. 4. A. Uptake in tissues at 24, 48 and 72 hours after administration of ^{111}In-DTPA-trastuzumab (Herceptin®) Fab and ^{111}In-DTPA-HuM195 Fab (72 h) in athymic mice bearing s.c. BT-474 human breast cancer xenografts. **B.** T/NT ratios at selected times post-injection. Tissues shown are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).

Tumor uptake for ^{111}In-trastuzumab Fab at 24, 48 and 72 hours was 5.2 ± 1.5, 8.4 ± 1.8 and 7.8 ± 0.7% ID/g. Tumor/blood (T/B) ratios increased more than 6 fold to 25.2:1 at 72 hours post-injection compared to that at 24 hours (4.2:1). This increase was mainly due to the diminished blood background rather than further uptake of ^{111}In-trastuzumab Fab in BT-474 tumors after 48 hours. The tumor uptake of ^{111}In labeled HuM195 anti-CD33 control Fab was 2.7 % ID/g and the T/B ratio was 6.3:1 at 72 hours. Both values were significantly lower than those of ^{111}In-trastuzumab Fab (t-test, $p<0.05$). These results demonstrated that ^{111}In-trastuzumab Fab specifically localized in HER-2/neu positive BT-474 tumors. In addition, we also examined the tumor uptake of ^{111}In-trastuzumab IgG at 72 h p.i. Despite the high tumor uptake (38.7 % ID/g), the T/B ratio was only 3.0:1 (not shown). These findings are consistent with reported observations that small antibody fragments, such as Fab, provide very high T/NT ratios due to their rapid blood clearance and therefore are more suitable for imaging purposes than intact IgG. Taken together, these results further suggest that a time delay of 48 to 72 h between injection of ^{111}In-labeled Fab fragments of trastuzumab (Herceptin®) and performance of radioguided surgery in patients may provide higher tumor/background ratios which could enhance the sensitivity and resolution of the technique. The use of a longer half-life radiolabel such as ^{111}In ($t_{½}$ 67 hours) would permit the time delay required.

Task 4: Conduct preclinical testing of radiopharmaceuticals in phantom models

Purchase of gamma detecting surgical probe. After consultation with Dr. Claire Holloway, our breast surgeon and co-investigator who will evaluate the probe clinically in the final year of the project regarding different commercially available surgical gamma probes, it was decided to purchase the C-Trak® analyzer and probe (Care Wise Medical Products, Morgan Hill, CA, **Fig. 5**). The probe was purchased to conduct preliminary experiments examining the sensitivity and resolution of detection of radioactivity in the radioguided surgery approach using phantom models of simulated DCIS lesions. The OmniProbe® consists of a NaI(Tl) crystal and

photomultiplier tube connected to a compact console containing an automatic analyzer (**Fig. 5 A**). The system is capable of detecting γ-rays with energy up to 364 keV, although it is optimized for measuring gamma-emissions of ^{99m}Tc (140 keV) and ^{111}In (173 and 247 keV). To reduce the effect of background radiation and allow for the highly directional detection of a radioactive source, the probe comes equipped a low-energy collimator for ^{99m}Tc and a medium-energy collimator for ^{111}In (**Fig. 5 B**). The system's operating parameters can be set and the detected radiation observed on the display. The analyzer has an internal battery power source, so it is not susceptible to electrical interference from other equipment in the operating room.

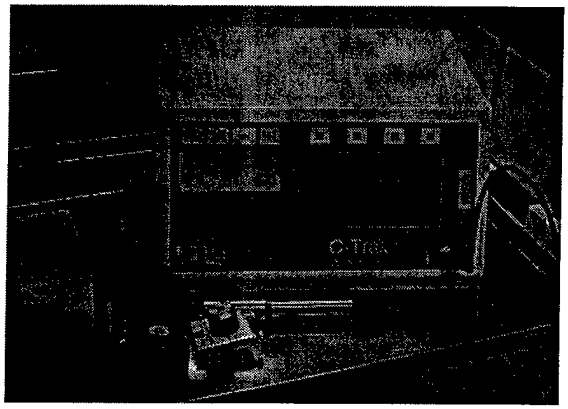


Fig. 5. A. Display unit and control panel for C-Trac® surgical guidance system (CareWise Medical Products).

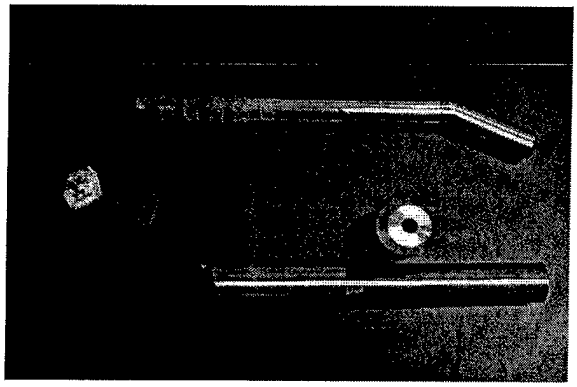


Fig. 5 B. OmniProbe® NaI (Tl) scintillation γ-detecting probe fillted with low energy collimator for ^{99m}Tc (upper) or medium energy collimoator for ^{111}In (lower). Also shown is radioactive ^{57}Co check source for system calibration.

To establish the operation and detection capabilities of the C-Trak® system prior to its use in radioguided surgery, a series of basic radiation detection experiments were conducted. An aluminum frame was constructed by our in-house Medical Engineering department so the probe could be moved in precise increments in the *x*, *y*, and *z* directions with respect to a radionuclide source to accurately determine the spatial resolution for detection of a DCIS lesion (**Fig 6**). Radionuclides such as ^{99m}Tc, ^{111}In and ^{123}I, were pipetted into small wells (inner diameter = 7 mm) of a rectangular polystyrene tissue culture plate to create phantoms that simulate a DCIS lesion. Other radionuclides, such as ^{57}Co, ^{133}Ba, and ^{129}I, supplied by the manufacturer as sealed point sources encased in a plastic planchet were also used to evaluate the C-Trac® probe system.

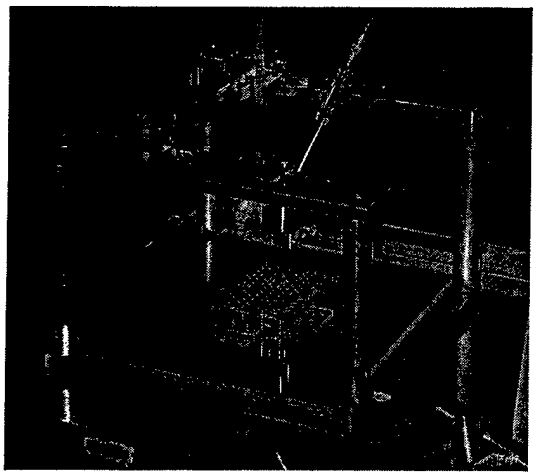


Fig. 6. Framework with attached OmniProbe® used to evaluate the sensitivity and spatial resolution of the C-Trac® surgical guidance system for detection of a simulated DCIS lesion. Simulated DCIS lesions were created using a phantom consisting of a plastic multiwell plate containing sources of radioactivity.

The first set of phantom experiments examined the intrinsic energy resolution of the probe system for different radionuclides. This is important because the greater the energy resolution, the greater the possibility to set appropriate energy windows to exclude scattered counts not originating from the lesion and thereby improve the spatial resolution of the probe. The energy resolution for ^{99m}Tc, ^{111}In, ^{57}Co, ^{123}I, ^{133}Ba, and ^{129}I was determined by setting a narrow energy window of 5 or 10 keV and increasing the threshold or lower level discriminator (LLD) in small increments throughout the range of the automatic analyzer (0 – 400 keV). The data were plotted to obtain a γ-spectrum. The energy (keV) of the photopeak was identified from the γ-spectrum and the energy resolution (percent of photopeak energy) was calculated as full width at half-maximum (FWHM). The FWHM for ^{99m}Tc or ^{111}In (22-25% and 22-30%, **Table 1**) compared favourably to FWHM values cited in the literature for other surgical gamma probe systems (21).

Table 4: Summary of energy resolution measurements for selected radionuclides.

Radionuclide	Energy Window Increment (keV)	Collimation	Photopeak Energy (keV)	Energy Resolution (FWHM)
^{99m}Tc	10	No	140	22%
	10	Yes	140	23-26%
	5	Yes	140	22%
^{111}In	10	Yes	172	23%
	10	Yes	247	22%
	5	Yes	172	23-30%
	5	Yes	247	23-30%
^{57}Co	5	Yes	122	22-25%

The next set of experiments examined the spatial resolution of the γ-detecting probe system for detecting a radioactive source. These studies are important in that they project the ability of the probe to discriminate a DCIS lesion and also identify the smallest size lesion which may be identified intraoperatively. Using the manufacturer's pre-defined energy threshold and window, the spatial resolution for ^{57}Co, ^{99m}Tc and ^{111}In was determined by mounting the probe in the holding bracket of the aluminum frame in order to move the probe overtop of a source in the *x*

and y directions, usually in 5 mm increments. The distance between the source and probe was adjusted (z direction) and the x and y measurements repeated. The data were plotted in both 2-D and 3-D maps from which the FWHM of the point spread function (PSF) was calculated. As expected, the symmetry of the radioactivity counts data relative to the position of the radionuclide source was exhibited. As the source-to-probe distance increased, the spatial resolution worsened. The results of these experiments also showed a decrease in counts (detected γ-emissions) in accordance with the Inverse-Square Law. The Inverse-Square Law states the radiation flux (I) is inversely proportional to the distance squared (d^2) from a radionuclide point source ($I \propto 1/d^2$) (22). Typical PSF's for ^{111}In at 7 mm or 17 mm above the source using either the medium energy ^{111}In collimator or the low energy ^{99m}Tc collimator are shown in **Fig. 7**. The lighter low energy collimator is intended for ^{99m}Tc and not ^{111}In, but it may nevertheless be useful from a practical point of view for intraoperative detection of ^{111}In, since it is much lighter than the medium energy collimator, and easier for the surgeon to handle. We were therefore interested to evaluate the effect the low energy collimator on spatial resolution for ^{111}In.

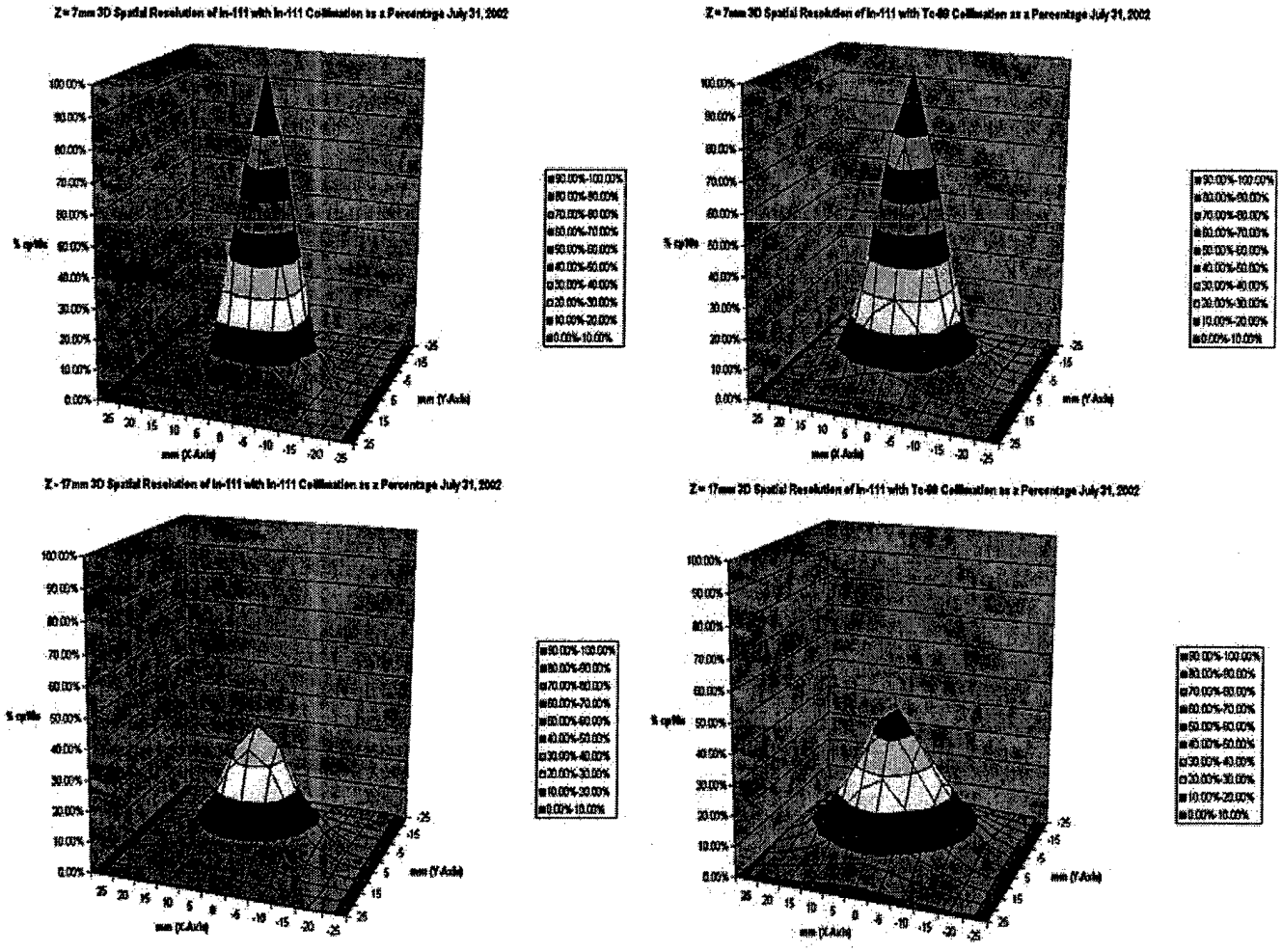


Fig. 7. 3-D spatial resolution showing the point spread functions for an ^{111}In source using the medium-energy collimator at 7 mm (top left) and 17 mm (bottom left) above the source and, similarly, using the low-energy collimator (right).

Spatial resolution deteriorated using the low energy collimator for ^{111}In as predicted because the higher-energy emissions of ^{111}In, particularly the 247 keV γ-photon were able to penetrate the comparatively thinner walls of the low energy collimator. Nevertheless more investigation is necessary to clearly establish whether or not the surgeon could use the lighter collimator for ^{111}In detection under clinical conditions without adversely sacrificing spatial accuracy.

The next set of experiments examined the ability of the probe system to discriminate between two sources of radioactivity placed at different distances from each other. This is important because DCIS may contain "skip" lesions, in which there can be one lesion separated from another by an area of normal breast tissue. Spatial resolution at varying distances between two radionuclide sources was investigated by pipetting small activities of ^{111}In into 7-mm diameter wells of a tissue culture plate (**Fig 6**). With appropriate collimation, the C-Trak® system demonstrated excellent spatial down to a 9-mm separation. The separation between the two sources was limited only by the 7-mm diameter of the collimators' apertures.

The next set of experiments examined the effects of attenuation by overlying soft tissue. These experiments are important because the sensitivity of intraoperative detection of DCIS lesions will be effected by attenuation of the signal by overlying normal breast tissue. Tissue attenuation was simulated by placing a known thickness of water between the collimated probe and the radionuclide source. Water was chosen to mimic adipose tissue found in the breast because the mass attenuation coefficient of water (μ_m = 1.116 cm^2/g for 140 keV) closely resembles that of adipose tissue (μ_m = 1.264 cm^2/g for 140 keV) (23). The thickness of water ranged between 0 and 100 mm. Interestingly, only minor differences were detected in the count rates obtained with water or air as the intervening substance (**Fig. 8**). This may be due to the geometry of the radioactive source (ie. not a point source) as well as the geometry of the detector (7 mm collimator aperture), which makes the inverse square law the overriding factor responsible for diminishing counts rather than attenuation by the intervening materials. These findings suggest that in the operating room environment, the probe will likely be sensitive enough to detect relatively deep-lying lesions.

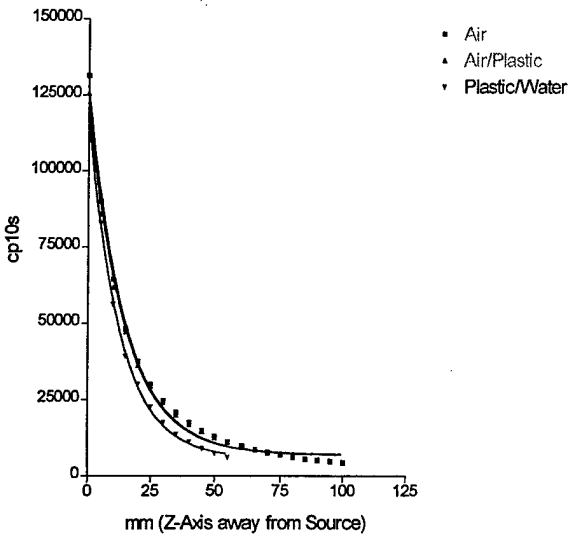


Fig. 8. Decrease in signal detected by the C-Trac® surgical guidance system as a function of increasing distance (z-axis) from the radioactive source using different intervening materials (air, plastic or plastic combined with water).

Repeated measurements of background radiation (no activity present in the vicinity of the probe) were used to calculate the Minimum Detectable Activity (MDA) according to the formula MDA $= 3(R_b/t)^{½}$, where R_b is the average of the background counts and t is the acquisition time in minutes. The MDA is defined as the smallest amount of radioactivity distinguishable from ambient background that can be quantified at a 95% confidence level. It is specific to a particular gamma-detection system and it varies with the radionuclide (22). The results in counts per minute (cpm) are summarized in **Table 5**.

Table 5. Minimum Detectable Activity (MDA) for the C-Trac® Surgical Guidance System.

Radionuclide	Minimum Dectectable Activity (MDA)
^{99m}Tc	6 cpm
^{111}In	7 cpm
^{57}Co	8 cpm

Finally, the effect of the geometric arrangement between the probe and the radionuclide source was tested. A point source of radioactivity emits gamma-rays in all directions, but even when the OmniProbe® is positioned directly overtop of the radionuclide source, it can theoretically, at best, detect only 50% of the gamma-rays. In reality, this counting efficiency is poorer because of properties of the NaI(Tl) crystal itself and the design of the collimator. **Table 6** summarizes the geometric efficiencies and intrinsic efficiencies (calculated by correcting the geometric efficiency for the abundance of the γ-photon) for selected radionuclides. The geometric efficiency has an impact on the amount of radiopharmaceutical required to be administered to the patient so that the OmniProbe® can sensitively detect DCIS lesions in the breast.

Table 6. Geometric and intrinsic efficiencies for the C-Trak® surgical guidance system.

Radionuclide	Detection Window	Geometric efficiency	Abundance of γ-Photon	Intrinsic Efficiency
^{57}Co	112-132 keV	2.9%	85.9%	3.4%
^{99m}Tc	130-170 keV	2.0%	87.9%	2.4%
^{111}In	150-300 kEv	2.1%	89.6% and 94.0%	2.3%

$$\text{Geometric Efficiency} = \frac{\text{cps} - \text{background cps}}{\text{disintegrations/sec}} \times 100\%$$

$$\text{Intrinsic Efficiency} = \frac{\text{cps} - \text{background cps}}{\text{disintegrations/sec} \times \gamma\text{-abundance}} \times 100\%$$

Definitions for geometric and intrinsic efficiency are described in reference 23.

PLANNED RESEARCH FOR YEAR 3 (2002-2003)

We intend to request modification of the original Statement of Work to continue with the development of a novel recombinant Fab' fragment of mAb CC49 containing an integrated radiometal binding site for ^{99m}Tc which we feel will address the issues of low immunoreactivity observed with enzymatically produced CC49 Fab fragments labeled with ^{99m}Tc described in this report. In the revised Statement of Work we would also like to include the development of a clinical quality radiopharmaceutical kit manufactured under Good Manufacturing Practises (GMP) for labeling CC49 Fab or trastuzumab (Herceptin®) Fab for a pilot Phase I testing in patients with DCIS in the final year of the project. The revised Statement of Work will require a one year extension in the end date of the contract to permit time for the clinical trial which is now scheduled for 2003-2004. The planned research for Year 3 and Year 4 described in the following sections take into account the request for the revised Statement of Work.

Task 5. Develop a novel recombinant Fab' fragment of monoclonal antibody CC49 containing an integrated radiometal binding site. A recombinant Fab' fragment of mAb CC49 containing an integrated radiometal binding site for ^{99m}Tc will be constructed, expressed in *Pichia pastoris*, purified and labeled with ^{99m}Tc as previously described. The immunoreactivity of the recombinant CC49 Fab' will be evaluated *in vitro* using bovine submaxillary mucin (BSM) and its tumor and normal tissue localization properties will be evaluated *in vivo* in the LS174T tumor xenograft model as previously described for enzymatically generated CC49 Fab'. Comparisons will be made with the enzymatically produced and recombinant Fab' fragments to determine if this approach has potential for generating a more immunoreactive and therefore more clinically useful radiopharmaceutical for radioguided surgery of DCIS.

Task 6. Develop and manufacture a GMP quality radiopharmaceutical kit for labeling Fab' fragments with ^{99m}Tc or ^{111}In. In order to provide a radiopharmaceutical suitable for clinical evaluation in a proposed pilot Phase I trial of radioguided surgery of DCIS, it will be necessary to develop and manufacture under Good Manufacturing Practices (GMP) conditions a radiopharmaceutical kit for labeling mAb CC49 or trastuzumab (Herceptin®) Fab' to high eficiency (>90%) with ^{99m}Tc or ^{111}In. The kit will consist of a single patient dose vial containing a sterile, pyrogen-free 1-mL solution of 0.5-1 mg of Fab' in a suitable bufer for labeling with ^{99m}Tc or ^{111}In. The final radiopharmaceutical will be prepared by simply adding the radionuclide to the vial, incubating for the required time and performing quality control testing. Health Canada requires that specifications for all raw materials, intermediates (kits) and final radiopharmaceutical product be established and that at least 3 separate batches be prepared and tested against these specifications. This information must be included in the Chemistry and Manufacturing section of a Clinical Trial Application (CTA) which is equivalent to an Investigational New Drug Application (IND) in the U.S. In 2002-2003, we will therefore manufacture the kit and establish all specifications and test pilot batches to obtain the required data for the CTA submission to Health Canada.

PLANNED RESEARCH FOR YEAR 4 (2003-2004)

Task 7 : Obtain all regulatory approvals for a pilot Phase I trial of radioguided surgery of DCIS. Early in the final year of the project (now 2003-2004), we will submit applications to the

institutional Research Ethics Board (REB) at Sunnybrook and Women's College Health Sciences Centre in Toronto, Health Canada and the Human Subjects Review Board of the U.S. Army for approval to conduct a Phase I clinical trial of radioguided surgery in patients with DCIS.

Task 8 : Conduct a Phase I clinical trial of radioguided surgery of DCIS. After obtaining the necessary regulatory approvals, a pilot Phase I clinical trial of radioguided surgery of DCIS will be conducted in approx. 12 patients at Sunnybrook and Women's College Health Sciences Centre in Toronto. A draft of the clinical trial protocol is currently being written.

KEY RESEARCH ACCOMPLISHMENTS

- Expanded the number of cases of DCIS, invasive breast cancer and benign breast disease tested for immunoreactivity with monoclonal antibody CC49 and trastuzumab (Herceptin®). Compared the reactivity of trastuzumab (Herceptin®) with the more commonly used CB11 or HercepTest® for evaluating expression of HER-2/neu.

- Completed preclinical testing of trastuzumab (Herceptin®) Fab fragments labeled with ^{111}In in athymic mice implanted subcutaneously with HER-2/neu positive BT-474 human breast cancer xenografts.

- Purchased a commercial gamma detecting surgical guidance system (C-Trac®, Carewise Medical Products) and fully evaluated the sensitivity and spatial resolution of the system using an in-house manufactured framework and phantom models simulating DCIS lesions.

- Constructed an expression vector for a recombinant Fab fragment of monoclonal antibody CC49 containing an integrated radiometal binding site for ^{99m}Tc, expressed the proteins in Pichia pastoris and completed preliminary characterization with respect to purity and immunoreactivity against bovine submaxillary mucin (a source of the TAG-72 antigen).

REPORTABLE OUTCOMES

Manuscripts

Tang Y., Wang, J., Kahn, H., Holloway C. and Reilly R.M. Imaging of HER-2/neu positive breast cancer xenografts in athymic mice using trastuzumab (Herceptin®) labeled with ^{99m}Tc or ^{111}In. (manuscript in preparation) 2002.

Abstracts

*Tang, Y., Wang, J., Holloway, C., Catzavelos,C., Sandhu, J., Hendler, A. and Reilly R.M. Construction of ^{125}I or ^{99m}Tc labeled Fab' fragments of monoclonal antibodies (mAbs) CC49 or Herceptin® for radioguided surgery of ductal carcinoma in situ of the breast (DCIS). Proc. Amer. Assoc. Cancer Res. 42: 700 [abstract 3764] 2001.

*Recipient of AACR Glaxo-Smith-Kline Scholar-in-Training Award for the abstract.

Tang, Y., Wang , J, Holloway, C., Kahn, H., Sandhu, J., Hendler, A and Reilly, R.M. Construction of ^{99m}Tc or ^{111}In-labeled Fab fragments of monoclonal antibody Herceptin for radioguided surgery of ductal carcinoma in situ of the breast. Presented at U.S. Army Breast Cancer Research Program Era of Hope conference, Orlando, FL, September 25-28, 2002.

Wong, K.K., Scollard, D.A., Tang, Y. and Reilly, R.M. An investigation of the detection properties of the CareWise C-Trak® intraoperative gamma-detecting OmniProbe. Presented at 2002 Summer Undergraduate Research Day, Faculty of Pharmacy, University of Toronto, Aug. 15, 2002.

Tang, Y., Yang, S., Gariepy, J., Chen, C. and Reilly, R.M. Novel recombinat Fab fragments of the TAG-72 monoclonal antibody CC49 containing an integrated radiometal binding site for radioimmunoguided surgery of DCIS. Accepted for presentation at the EORTC/NCI/AACR Meeting on Molecular Targets and Cancer Therapeutics, Frankfurt, Nov. 19-22, 2002.

Presentations

Reilly, R.M. Molecular imaging of cancer. Presented at the Faculty of Pharmacy, University of Toronto, April 10, 2002.

Reilly, R.M. Molecular imaging and targeted radiotherapy of cancer: Past, present and future. Presented at Department of Medical Biophysics seminar, Sunnybrook and Women's College Health Sciences Centre, Toronto, ON, July 4, 2001.

Reilly, R.M. Molecular imaging of breast cancer. Presented at Imaging Network Ontario Symposium, Toronto, ON, October 20, 2001.

Applications for Funding Based on Research

Tang, Y. (predoctoral candidate) and Reilly, R.M. (mentor). Novel recombinant Fab fragments of the TAG-72 monoclonal antibody CC49 containing an integrated radiometal binding site for radioimmunoguided surgery of DCIS. U.S. Army Breast Cancer Research Program Pre-doctoral training award. $ 30,000/year 2003-2004 (applied).

Tang, Y. (dissertation research candidate) and Reilly, R.M. (supervisor). Novel recombinant Fab fragments of the TAG-72 monoclonal antibody CC49 containing an integrated radiometal binding site for radioimmunoguided surgery of DCIS. Susan G. Komen Breast Cancer Foundation. PhD Dissertation Research award. $ 15,000/year 2003-2004 (applied).

CONCLUSIONS AND FUTURE RESEARCH

The conclusions of the research conducted in Year 2 (2001-2002) of the project are as follows:

1. Monoclonal antibodies CC49 and trastuzumab (Herceptin®) are potential candidates for constructing radiopharmaceuticals for radioguided surgery of DCIS. CC49 reacts with about

60% of DCIS specimens, whereas trastuzumab (Herceptin®) reacts with about 20%. The higher immunoreactivity of CC49 suggests that this is perhaps the most promising candidate.

2. Fab fragments of monoclonal antibody CC49 and trastuzumab (Herceptin®) were generated by enzymatic methods. Trastuzumab (Herceptin®) Fab fragments exhibited preserved immunoreactivity against HER-2/neu positive SK-BR-3 human breast cancer cells *in vitro* whereas Fab fragments of CC49 exhibited a 100-fold decrease in immunoreactivity against TAG-72 in vitro using ELISA assays. Trastuzumab (Herceptin®) Fab labeled with ^{99m}Tc or ^{111}In localized specifically in HER-2/neu positive BT-474 human breast cancer xenografts in athymic mice achieving a tumor/blood ratio > 25:1 at 72 hours post-injection.

3. A recombinant Fab fragment of monoclonal antibody CC49 containing an integrated radiometal binding site may address the problem of decreased immunoreactivity. Due to the higher proportion of DCIS specimens reactive with monoclonal antibody CC49, this would be a worthwhile strategy to explore further.

4. The C-Trac® gamma surgical guidance system (CareWise Medical Products) has sufficient sensitivity and spatial resolution for intraoperative detection of radiopharmaceuticals targeted to DCIS lesions based on phantom studies.

In future research, we intend to further explore the feasibility of creating a recombinant CC49 Fab fragment with an integrated radiometal binding site for ^{99m}Tc. We would also like to develop a radiopharmaceutical kit manufactured under Good Manufacturing Practices (GMP) conditions for labeling CC49 or trastuzumab (Herceptin®) Fab fragments with ^{99m}Tc or ^{111}In for evaluation in a pilot Phase I clinical trial of radioguided surgery of DCIS in patients in the final year of the project (2003-2004).

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