PRE-TARGETED RADIOIMMUNOTHERAPY WITH STREPTAVIDIN-CC49 MONOCLONAL ANTIBODY AND ⁹⁰Y-DOTA-BIOTIN

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmaceutical Sciences
University of Toronto

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Master of Science 1997, Rommel J. Domingo
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ABSTRACT

In this study pre-targeted radioimmunotherapy (RIT) of athymic mice bearing subcutaneous LS174T human colon cancer xenografts was evaluated using streptavidin-CC49 and yttrium-90 (⁹⁰Y)-DOTA-biotin. The biodistribution of DOTA-biotin, labeled with indium ¹¹¹ (¹¹¹In), was first investigated in BALB/c mice to determine its suitability for pre-targeting strategies. Indium ¹¹¹-DOTA-biotin was rapidly eliminated from blood and normal tissues. A dosing schedule was then determined by evaluating different time intervals between the administration of streptavidin-CC49 and DOTA-biotin and doses of DOTA-biotin in mice bearing LS174T colorectal xenografts. Comparison of 40 and 72 hour time intervals indicated that the 40 hour time interval would be the most effective for the pre-targeted RIT study. A comparison of 1, 20, 40, and 150 µg doses of DOTA-biotin indicated that 40 µg nearly achieved maximal uptake of DOTA-biotin in the tumour. Using the dosing schedule developed, the therapeutic efficacy and toxicity of pre-targeted RIT with 900 µCi of ⁹⁰Y-DOTA-biotin was evaluated in athymic mice bearing LS174T colorectal xenografts. Considerable suppression in tumour growth was observed in 1/6 treated mice. No apparent bone marrow toxicity and deterioration in health were observed in treated mice. In summary, pre-targeted RIT with streptavidin-CC49 and ⁹⁰Y-DOTA-biotin demonstrated some promise as an effective and safe approach in the treatment of colorectal cancer.
ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to Professor Ray Reilly whose guidance, encouragement, and kindness have made the past two years a very rewarding and memorable experience. Moreover, his fairness, approachability, and outgoing nature made working at The Toronto Hospital very comfortable and enjoyable. I will always be grateful to Professor Reilly, for his teaching and exemplary research have helped me accomplish my educational goals.

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$^{67}$Cu copper 67
$^{90}$Y yttrium 90
$^{111}$In indium 111
$^{125}$I iodine 125
$^{131}$I iodine 131
$^{188}$Re rhenium 188
$^{211}$At astatine 211
$^{212}$Bi bismuth 212
$\alpha$ alpha
$\beta$ beta
$\gamma$ gamma
ABMT autologous bone marrow transplantation
BSM bovine submaxillary mucin
CDR complementarity determining region
CEA carcinoembryonic antigen
CSFs colony stimulating factors
DOTA 1, 4, 7, 10-tetraazacyclododecane-N, N', N", N'''-tetraacetic acid
DTPA diethylenetriaminepentaacetic acid
ECIA extracorporeal immunoadsorption
gy grays
HAMA human anti-mouse antibody
HASA human anti-streptavidin antibody
HPLC high pressure liquid chromatography
iv intravenous
ip intraperitoneal
IL-1 interleukin-1
IL-2 interleukin-2
$K_a$ binding affinity constant
kDa kilodaltons
mAb monoclonal antibody
MRUs molecular recognition units
pi post-injection
RES reticuloendothelial system
RIT radioimmunotherapy
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sulfo-SMCC sulfo[125I]labeled 4-(N-maleimideomethyl)cyclohexane-1-carboxylate
tag-72 tumour-associated glycoprotein 72
US FDA United States Food and Drug Administration
WBC white blood cells
XRT external radiation therapy
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INTRODUCTION

Radioimmunotherapy (RIT) is a form of cancer treatment in which radiolabeled antibodies are administered to a patient to selectively deliver ionizing radiation to the lesion. The idea is to deliver a sufficient radiation dose to the tumour tissues in order to achieve suppression of tumour growth, or at best, eradication of the tumour, while normal tissues are spared toxicity. Radioantibodies are selectively taken up in tumour tissues by binding to specific antigens or receptors located on the surface of the cancer cells. These antigens are not truly specific to cancer cells, but rather, are tumour-associated. They are highly overexpressed in tumour tissues compared to normal tissues, which in most cases, provides for a sufficient gradient that achieves antibody localization in the tumour. Many anti-tumour antibodies are pan-carcinomic, capable of recognizing more than one tumour-associated antigen. In many cases, a given cancer can also be targeted with more than one type of antibody.

The earliest reports of using antibodies to treat cancer were published in 1895 by Hericourt and Richet (Hericourt and Richet, 1895) and in 1906 by Paul Ehrlich (Himmelweit, 1957). These early attempts, however, were neither successful or reproducible, and the properties of the antibodies were unknown. The idea, however, of treating cancer with antibodies was launched. In the late 1940's a method to label proteins with iodine 131 ($^{131}$I) was developed (Eisen and Keston, 1950) and one decade later, clinical trials of RIT began. Beierwaltes (Beierwaltes, 1974) administered $^{131}$I-labeled rabbit antibodies to 14 patients with metastatic melanoma and achieved a pathologically documented response in one patient. Thereafter, polyclonal antibodies against tumour-associated antigens such as, the prototypical carcinoembryonic antigen (CEA), were developed, used in patients, and produced some favourable responses (Ettinger et al., 1979; Ettinger et al., 1982). The field of RIT was revolutionized in 1975 when Köhler and Milstein (Köhler and Milstein, 1975) introduced hybridoma technology, a method that produces large quantities of murine monoclonal antibodies (mAbs) with high, pre-defined specificity. Since then, numerous mAbs against a
wide variety of human cancers (Reisfield and Cheresh, 1987) have been developed and correspondingly, research efforts in RIT have increased tremendously.

Since 1975 hundreds of papers dealing with RIT have been published. More than twenty years of intense research in the field have yielded important findings, which can be summarized as follows (Stigbrand et al., 1996): i) the amount of radiolabeled antibody localized in solid tumours of patients is very small at <0.1% of the injected activity; ii) as a result of the low antibody uptake in tumours, the total absorbed radiation dose delivered to the tumour is also very small, typically <2 gray (Gy); iii) increasing the administered activity in order to increase the radiation dose delivered to the tumour is limited by bone marrow toxicity, caused primarily by passive irradiation of the bone marrow by the circulating radioantibodies; iv) the immunogenicity of murine mAbs triggers the production of human anti-mouse antibodies (HAMA) in patients, thereby limiting the number of treatments they can undergo; v) significant tumour regression or complete responses are often achieved in animal models, while mostly partial and transient responses are achieved in patients with certain solid tumours; and vi) hematological malignancies such as, B-cell lymphomas, are generally more responsive to RIT than certain solid tumours.

Monoclonal antibodies themselves are attractive candidates for cancer therapy because of the specificity they can have for tumour tissue. They are considered to be relatively safe, with toxicity limited only to transient immune-mediated or allergic-type reactions (Dillman et al., 1986). Allergic reactions such as hives are uncommon and severe anaphylaxis is rare (Kuzel and Duda, 1992). Other reactions include, fever, chills, dyspnea, hypotension, and shortness of breath (Goldenberg, 1994; Kuzel and Duda, 1992). Currently, there is no mAb preparation approved for clinical RIT. Two mAb products, however, Satumomab pendetide (Oncoscint CR/OV®) and Arcitumomab (CEA-Scan®) have been approved by the United States Food and Drug Administration (US FDA) for diagnostic radioimmunoimaging of prostate and colorectal cancer, respectively. Radioimmunoimaging is a cancer imaging modality analogous to that of RIT, in which radiolabeled mAbs are targeted to tumours, that are
subsequently visualized by a gamma camera. Radioimmunotherapy has suffered in that only sub-therapeutic doses of radiation are achieved in the tumour, while high whole body irradiation has regularly resulted in bone marrow toxicity and correspondingly, poor therapeutic indices. Moreover, the immunogenicity of murine mAbs precludes repeated courses of therapy, since HAMA responses can significantly alter the biokinetics of the administered radioantibody. Promising results have been obtained from animal RIT studies (Table 1), however, clinical results have yet to demonstrate a similar degree of success (Table 2). Advancements in molecular biology and in radiochemistry, along with the development of innovative tumour targeting methods are paving the way for new and alternative strategies that can potentially overcome the obstacles currently limiting the clinical success of RIT. For example, one innovative strategy to reduce bone marrow toxicity involves pre-targeting, in which a multi-step approach utilizing (strept)avidin and biotin is employed (see: "Pre-targeting with the (Strept)avidin-biotin System"). Reducing bone marrow toxicity is key to improving the clinical success of RIT because it may also allow larger amounts of radioactivity to be administered to the patient, which in turn, may possibly lead to improved radiation doses delivered to tumour tissues.
Table 1. Summary of various animal radioimmunotherapy trials.

<table>
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<tr>
<th>Cancer Type</th>
<th>Cell Line</th>
<th>mAb; Specificity</th>
<th>Radionuclide</th>
<th>Administered Dose, Route</th>
<th>Therapeutic Responses</th>
<th>Toxicity</th>
<th>Reference</th>
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<td>Mammary</td>
<td>Obtained from patient biopsy</td>
<td>BW495/36</td>
<td>131I</td>
<td>200 μCi, iv</td>
<td>Mean decrease of 50% and 88% in tumour diameter and tumour volume, respectively</td>
<td>&lt;30% decrease in leukocyte and platelet counts</td>
<td>Senekowitsch et al., 1989</td>
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<tr>
<td>Mammary and ovarian</td>
<td>SKOV3, SKBR3</td>
<td>4D5, 7C2; anti-HER-2/neu oncoprotein</td>
<td>131I</td>
<td>400-700 μCi, iv</td>
<td>Marked inhibition of tumour growth</td>
<td>Myelosuppression</td>
<td>De Santes et al., 1992</td>
</tr>
<tr>
<td>T-cell lymphoma</td>
<td>AKR/J, SL2, AKR/Cum SL1</td>
<td>31E6.4; anti-Thy-1.1</td>
<td>131I</td>
<td>1500 μCi, iv</td>
<td>Absence of palpable tumour in 71% of mice</td>
<td>100% of treated mice died from bone marrow aplasia</td>
<td>Badger et al., 1986.</td>
</tr>
<tr>
<td>Colorectal</td>
<td>GW-39</td>
<td>NP-2; anti-CEA</td>
<td>90Y</td>
<td>10-100 μCi, iv</td>
<td>Up to 71% inhibition of tumour growth at 50 μCi dose</td>
<td>Myelosuppression at 20 and 50 μCi doses with lethality at doses &gt;50 μCi</td>
<td>Sharkey et al., 1988</td>
</tr>
<tr>
<td>LS174T</td>
<td>ZCE025; anti-CEA</td>
<td>90Y</td>
<td>40-160 μCi, ip</td>
<td></td>
<td>93% reduction in tumour mass</td>
<td>30-85% depletion of bone marrow and splenic hypoplasia ND</td>
<td>Esteban et al., 1990</td>
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<tr>
<td>LS174T</td>
<td>ZCE025; anti-CEA</td>
<td>90Y</td>
<td>120 μCi, ip</td>
<td></td>
<td>98% reduction in tumour growth rate</td>
<td>Severe leukopenia and lethality</td>
<td>Buras et al., 1990a</td>
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<td>SW 948</td>
<td>CO17-1A</td>
<td>90Y</td>
<td>100-200 μCi/25 g body weight, iv</td>
<td></td>
<td>Up to 87% reduction in tumour volume</td>
<td></td>
<td>Lee et al., 1990</td>
</tr>
</tbody>
</table>

Abbreviations: 131I, iodine 131; 90Y, yttrium 90; iv, intravenous; ip, intraperitoneal; CEA, carcinoembryonic antigen; ND, not determined.
Table 2. Summary of various clinical radioimmunotherapy trials.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>mAb; Specificity</th>
<th>Radionuclide</th>
<th>Administered Dose, Route</th>
<th>Therapeutic Responses</th>
<th>Toxicity</th>
<th>HAMA Responses</th>
<th>Reference</th>
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<tr>
<td>B-cell lymphoma</td>
<td>Lym-1</td>
<td>$^{131}$I</td>
<td>30-60 mCi at 2-6 week intervals until death, HAMA, or until 300 mCi in total, iv</td>
<td>10/18 PR and CR, 3/18 SD, 4/18 NR, 1/18 PD</td>
<td>Hypotension, transient fever and chills Thrombocytopenia in 2/18 patients</td>
<td>2/18 patients</td>
<td>DeNardo et al., 1990</td>
</tr>
<tr>
<td>Ovarian</td>
<td>MB-1; anti-CD37, HMFG1, HMFG2, AUA1, H17E2; anti-human milk fat globule</td>
<td>$^{131}$I</td>
<td>250-482 mCi, iv, 20-158 mCi, ip</td>
<td>4/4 CR</td>
<td>Myelosuppression in 4/4 patients Nausea, vomiting, mild to moderate abdominal pain, and flu-like symptoms Myelosuppression in all patients receiving &gt;100 mCi dose</td>
<td>ND</td>
<td>Bernstein et al., 1990 Stewart et al., 1989</td>
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<td>Colorectal</td>
<td>Chimeric B72.3; anti-TAG-72</td>
<td>$^{131}$I</td>
<td>18-36 mCi/m², iv</td>
<td>4/12 SD, 8/12 PD, 1/12 MR</td>
<td>Hypotension, transient fever and nausea Myelosuppression in patients receiving &gt;27 mCi/m² Myelosuppression in patients receiving &gt;80 mCi/m²</td>
<td>9/12 patients</td>
<td>Meredith et al., 1992a</td>
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<td></td>
<td>A33</td>
<td>$^{131}$I</td>
<td>30-90 mCi/m², iv</td>
<td>3/23 MR</td>
<td>Myelosuppression in patients receiving &gt;27 mCi/m² Myelosuppression in patients receiving &gt;80 mCi/m²</td>
<td>23/23 patients</td>
<td>Welt et al., 1994</td>
</tr>
<tr>
<td></td>
<td>CC49; anti-TAG-72</td>
<td>$^{131}$I</td>
<td>75 mCi/m², iv</td>
<td>3/15 SD</td>
<td>Hypotension, transient fever and chills, and nausea Myelosuppression</td>
<td>12/15 patients</td>
<td>Murray et al., 1994</td>
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Abbreviations: $^{131}$I, iodine 131; iv, intravenous; ip, intraperitoneal; TAG-72, tumour-associated glycoprotein 72; ND, not determined; PR, partial response; CR, complete response; NR, no response; MR, mixed response; SD, stable disease; PD, progressive disease; HAMA, human anti-mouse antibodies.
RADIOIMMUNOTHERAPY CONCEPTS
Factors Influencing Tumour Uptake of Antibodies

Site of Administration

The site of administration may be important in the targeting of antibodies to tumours. Most applications have been by the intravenous (iv) route, which is ideal for targeting hematological or blood borne cancers. However, for compartmentalized cancers such as, ovarian cancer and colorectal cancer, intraperitoneal (ip) administration may be the most appropriate for it provides more direct contact between the antibodies and the cancer cells. Using B72.3 mAb labeled with $^{131}$I, researchers have shown that ip administration to patients with peritoneal carcinomatosis can achieve improved tumour targeting as compared to iv administration (Colcher et al., 1987; Larson et al., 1991a). In addition, targeting tumours residing in the lymphatics may be best achieved by local interstitial administration (Goldenberg, 1991).

Antibody Properties

The antibodies primarily used in RIT are murine IgG mAbs, which have a molecular weight of 150 kilodaltons (kDa), when in the intact form. The intact IgG consists of two identical heavy chains that weigh 53 kDa each and two identical light chains, each weighing 22 kDa (Figure 1). The biological functions of the antibody are determined by the constant regions, while the specificity for antigens is determined by the variable regions. The large molecular weight of the intact IgG antibody impedes its extravasation into tumours and its elimination from the blood. However, smaller molecular weight mAb derivatives such as, Fab, F(ab')$_2$, and scFv fragments and molecular recognition units (MRUs), have been developed (Figure 2). These smaller molecular weight derivatives retain the antigen binding specificity of the intact IgG mAb, and because of their smaller size, can achieve a more homogeneous tumour distribution, a faster rate of tumour uptake, and a faster rate of elimination from the blood and normal tissues (Yokota et al., 1992). A disadvantage, however, to using these smaller molecular weight derivatives is that they can have reduced binding affinity and avidity.
Figure 1. Structure of IgG antibody.

**Legend:**
- $V_1$: variable region; light chain
- $C_1$: constant region; light chain
- $V_{H1}$: variable region; heavy chain
- $C_{H1}$: constant region; heavy chain
- S—S: disulphide bridges
- CDRs: complementarity determining regions
compared to the intact mAb (Milenic et al., 1991; Yokota et al., 1992). This factor, combined with their rapid blood clearance, causes these mAb derivatives to have both a lower absolute uptake and a shorter residence time in the tumour compared to the intact antibody (Buchsbaum, 1995a).

Murine mAbs are foreign to the human immune system, and therefore, are immunogenic and capable of triggering HAMA responses in patients. Human anti-mouse antibodies responses can be generated either against the constant regions (anti-isotypic antibodies) or the variable regions (anti-idiotypic antibodies) of murine mAbs. Patients can elicit a HAMA response as early as 2 weeks following the administration of a single dose of murine mAb (Pimm et al., 1985), which only becomes problematic after repeated administrations of the foreign protein. Following repeated administrations, HAMA form immune complexes with circulating murine mAbs, which are then rapidly sequestered and degraded by the reticuloendothelial system (RES), i.e., liver and spleen, resulting in reduced antibody uptake in the tumour (Pimm et al., 1985). Toxicity from HAMA is rarely observed, but with repeated, large doses of murine mAb, the immune complexes formed could theoretically lead to serum sickness and end-organ damage (Jurcic et al., 1994). Fab, F(ab')2, and scFv fragments are less immunogenic than intact murine mAbs since they lack large or entire portions of the constant regions, the regions primarily responsible for triggering immune responses. Other mAb variants less immunogenic than the intact form include, intact humanized and intact chimeric mAbs (Figure 2). Humanized mAbs are composed of the human antibody framework, onto which, murine complementarity determining regions (CDRs) directed against the tumour-associated antigen are grafted. The CDRs are sequences of amino acids located on the variable regions of IgG antibodies that mediate antigen binding (Figure 1). Chimeric mAbs, which are less human in nature, consist of a murine variable region fused to a human constant region. Despite the reduction or elimination of the murine constant regions, HAMA responses, in the form of anti-idiotype reactions, against the mAb fragments and the chimeric and humanized mAbs can occur.
Figure 2. Generation of mAb fragments, chimeric mAb, and humanized mAb.

I. Molecular recognition units (MRUs) derived from complementarity determining regions (CDRs)

II. Murine CDRs grafted onto human IgG framework

III. Murine variable regions fused to human constant regions

IV. Enzymatic digestion by papain

V. Enzymatic digestion by pepsin

VI. Variable regions from the heavy and light chains joined by a peptide linker
Antibody preparations with the highest possible immunoreactivity are desirable. The immunoreactivity represents the fraction of the antibody preparation capable of binding to its target antigen. Current hybridoma technology is capable of producing large amounts of pure and specific mAb with 100% immunoreactivity (Stigbrand et al., 1996). According to results from in vitro studies and experimental systems, higher mAb binding affinity correlates directly with higher tumour uptake (Colcher et al., 1988). In contrast, some human studies (Gallinger et al., 1993; Divgi et al., 1994) show no such advantage to using mAbs with higher binding affinity.

**Metabolism of Antibodies**

Antibodies are catabolized in lysosomes following internalization by cells expressing Fc receptors (Wagle et al., 1978) or cells expressing the target antigen (Press et al., 1996). Binding of the antibody to Fc receptors via its Fc region is required for internalization by the Fc receptor-bearing cells. The primary site of catabolism of antibodies and other large macromolecules is the liver, where internalization is largely mediated by Fc receptors on Kupffer cells and hepatocytes. Similarly, the reticuloendothelial cells of the spleen and other Fc receptor-bearing cells also internalize and catabolize antibodies. Reducing the non-specific uptake of antibodies by Fc receptors can enhance the amount of administered antibody available for tumour localization, which in turn may lead to increased tumour uptake. In some instances, administration of unlabeled antibodies prior to the administration of the radiolabeled mAb may block the Fc receptors (Perkins and Pimm, 1991).

**Tumour Physiology**

The physiology of solid tumours imposes a variety of barriers, on a micro- and macroscopic level, that prevent antibody uptake in tumours from reaching optimum levels and achieving homogeneous distribution. Tumour characteristics such as, vascularity and vascular permeability, can limit antibody uptake to only well-perfused areas of a tumour. Although
many tumours may have increased vascular permeability compared to normal tissues, areas of high interstitial pressure within the tumour can oppose the influx of antibodies (Shockley et al., 1992a). Tumours exhibit a decrease in perfusion with increasing size (Dvorak et al., 1991), and therefore, demonstrate an inverse relationship between antibody uptake and tumour size (Hagan et al., 1986; Williams et al., 1988). In a study by Colcher et al. (1988), up to 3-fold more specific antibody localized in LS174T colorectal xenografts weighing 40-100 mg, grown in athymic mice, compared to similar tumour xenografts weighing 400-700 mg.

Not all tumour cells in the tumour express the tumour-associated antigen, which in turn, leads to heterogeneous distribution of antibody throughout the tumour. Higher tumour antigen density can result in greater accretion of antibody, however, tumour localization has been achieved even when only 15% of the tumour cells express the target antigen (Doerr et al., 1990). Shockley et al. (1992b) demonstrated in a human melanoma xenograft model that tumour uptake of antibody correlates directly with the level of antigen expression in the tumour. Moreover, in certain tumours, up-regulation of antigen expression induced by biological response modifiers such as, interferon, correlated with increased antibody uptake (Greiner et al., 1984). However, others have also suggested that targeting highly antigen-rich tumours with high affinity mAbs can result in an even more heterogeneous antibody distribution throughout the tumour. It is hypothesized that in such tumours, high affinity mAbs that extravasate from the tumour capillaries bind primarily to the antigen-positive cells directly adjacent to the vasculature, creating a "binding site barrier". The "binding site barrier" is not a physical barrier caused by the formation of mAb-antigen complexes adjacent to the tumour vasculature. Rather, the "binding site barrier" is a phenomenon, in which the formation of mAb-antigen complexes decreases the number of free mAb molecules that can diffuse into the deeper regions of the tumour. Hence, antibody distribution in the tumour becomes heterogeneous, with pronounced binding occurring only adjacent to the tumour vasculature (Fujimori et al., 1989; Juweid et al., 1992). Antigen shedding from the surface of tumour cells into the circulation can also adversely affect tumour targeting. Shedding of tumour antigen into
the circulation can lead to the formation of mAb-antigen immune complexes in the vascular compartment and subsequently, which can subsequently undergo rapid sequestration and degradation by the RES (Ullén et al., 1995).

Considerations for Radioimmunotherapy

In the preceding sections, various biological and non-biological variables that can affect antibody uptake in tumours were discussed. These same variables are applicable to radioimmunoimaging and RIT. In order to highlight the complexities of RIT, the importance of these and other variables on RIT outcomes are discussed in the following sections.

Antibody Properties

Radioimmunotherapy is a form of systemic therapy that inevitably results in some radiation burden to normal tissues. Since intact IgG mAbs display very slow elimination from the blood (α-phase half-life of 11–15 hours; β-phase half-life of 18–44 hours) (Perkins and Pimm, 1991), non-tumour tissues can be exposed to high doses of radiation for prolonged periods of time as radiolabeled mAb remains in the blood and normal tissues. However, administration of smaller molecular weight mAb variants such as, Fab, F(ab')2, and scFv fragments and MRUs, can decrease the radiation burden to normal tissues because of their rapid elimination from blood (Stigbrand et al., 1996). Of the mAb fragments, scFv fragments (25 kDa) display the fastest elimination from blood, followed by Fab fragments (50 kDa), and then by F(ab')2 fragments (100 kDa) (Stigbrand et al., 1996; Henry et al., 1992). Fab and scFv fragments have also demonstrated high renal uptake via reabsorption through the proximal tubules (Behr et al., 1995; Kobayashi et al., 1996; Arend and Silverblatt, 1975), and therefore, can result in high radiation doses to the kidneys. Renal uptake of these fragments, however, can be significantly reduced through pre-administration of renal uptake blockers such as, L-lysine (Kobayashi et al., 1996; Pimm, 1995). Apart from the improved pharmacokinetics, mAb fragments may also offer improved biodistribution since the lack of a
constant region would reduce Fc receptor-mediated uptake by the liver and spleen. In a comparative study by Stein et al. (1994), liver and spleen uptake of intact 131I-RS7 mAb in tumour-bearing mice was at least 5- to 6-fold greater than that of 131I-RS7 F(ab')2. As a result, the radiation doses delivered to the liver and spleen were 19- and 18-fold higher, respectively, for intact 131I-RS7 mAb. In contrast, Lane et al. (1994) did not find a significant difference between the liver uptake of intact anti-CEA 131I-ASB7 mAb and that of 131I-A5B7 F(ab')2.

Since mAb fragments can display shorter residence times and lower absolute uptake in the tumour compared to intact mAbs, the radiation dose delivered to the tumour by mAb fragments could correspondingly, be lower as well. Wahl et al. (1983) compared the uptake of intact anti-CEA mAb to that of anti-CEA Fab and F(ab')2 fragments in tumour xenografts grown in athymic mice. The authors observed the tumour uptake of intact mAb to be 2.4-fold and 26-fold higher than that of the F(ab')2 and Fab fragments, respectively, at 48 hours following antibody administration. Similar results were obtained by Stein et al. (1994), who observed an approximately 2-fold greater uptake of intact RS7 mAb compared to RS7 F(ab')2 in tumour xenografts, at 24 hours. RS7 F(ab')2 fragments, however, displayed a much faster elimination from blood than intact RS7 mAb. Differences in absolute tumour uptake and in elimination from blood between RS7 mAb and RS7 F(ab')2 corresponded with similar differences in the radiation doses delivered to these tissues. At equivalent amounts of activity administered (25 μCi), 131I-RS7 mAb delivered radiation doses to the tumour and blood that were 4.4- and 20.8-fold higher, respectively, compared to 131I-RS7 F(ab')2. Despite having a lower uptake in tumours, F(ab')2 fragments are expected to demonstrate superior anti-tumour effects and less toxicity compared to intact mAbs, because of their faster rate of uptake in tumour, their more homogeneous tumour distribution, and their shorter biological half-life. While only 250 μCi of 131I-RS7 mAb was sufficient to achieve a 5 week suppression in tumour growth, as much as 1 mCi of 131I-RS7 F(ab')2 was required to achieve a comparable response. At equitoxic doses, however, 131I-RS7 F(ab')2 was expected to yield greater therapeutic efficacy than 131I-RS7 mAb. Escalating the dose of 131I-RS7 F(ab')2 by 50% to
1.5 mCi produced an 8 week suppression in tumour growth without any lethality to the animals. On the other hand, a similar dose escalation of 50% of $^{131}I$-RS7 mAb to 375 μCi, resulted in death of all the animals.

In cancer patients undergoing external radiation therapy (XRT), most normal tissues can tolerate higher cumulative doses of external beam radiation when they are given as several smaller fractions that are separated by sufficient time for repair, compared to one single dose (Nias, 1990). Moreover, fractionated RIT has been reported to be more efficacious and less toxic than the traditional single dose RIT. Animal models of fractionated RIT have demonstrated that larger, fractionated doses of $^{131}I$-labeled antibody achieve a greater degree of tumour regression compared to the administration of single maximally tolerated doses (Schlom et al., 1990; Buchsbaum et al., 1990). Schlom et al. (1990) reported that 60% of tumour-bearing mice died when treated with a single dose of 600 μCi of $^{131}I$-mAb, whereas 2 injections of 300 μCi a week apart resulted in death in only 10% of the animals. Fractionated RIT in patients, however, is limited by the HAMA response, particularly when intact murine mAbs are administered. Despite their decreased immunogenicity, mAb fragments and chimeric and humanized mAbs may not necessarily be more suitable for fractionated RIT than intact murine mAbs since these variants are still capable of inducing anti-idiotypic HAMA responses. In a dose fractionation study with colorectal cancer patients, 9/12 patients treated with chimeric $^{131}I$-B72.3 mAb developed an antibody response to the chimeric antibody (Meredith et al., 1992a). However, it was not determined whether the antibody response was an anti-idiotype reaction. Two of those nine patients produced such high amounts of antibody against the chimeric B72.3 mAb, that rapid clearance and excretion of chimeric B72.3 mAb occurred, consequently resulting in a 6-fold reduction in the whole body radiation dose received from a second course of treatment. In the study, no correlation between tumour response and antibody response was observed. Increased radiation doses to the liver and spleen can result from the formation of immune complexes with HAMA. On the other hand, HAMA may also protect
patients from bone marrow toxicity by reducing the radiation dose delivered to the blood (Stewart et al., 1989).

Interestingly, anti-idiotype HAMA responses may actually produce an anti-tumour effect in some cases. Anti-idiotype antibodies can mimic the original tumour-associated antigen, thereby potentially acting as a vaccine and immunizing the patient against the cancer associated with that antigen. In a clinical radioimmunoimaging trial, 7/32 patients that were repeatedly administered doses of murine mAb developed a strong anti-idiotypic HAMA response and surprisingly, displayed complete remission or stable disease for a period of 4-42 months (Baum et al., 1994).

**Radionuclides**

Radioisotopes suitable for RIT are listed in Table 3. It is very important to match the physical half-life of the radionuclide to the *in vivo* pharmacokinetics of the antibody to help ensure that sufficient doses and dose rates for cell sterilization are delivered to the tumour. If the half-life of the radionuclide is too short, then most of the decay would have occurred before tumour uptake of the antibody reaches maximum levels. Moreover, at equal radioactivity concentrations in the tumour, radionuclides with a long half-life will deliver a lower absorbed dose rate than those with a shorter half-life (Mausner and Srivastava, 1993). It has been suggested that using a radionuclide with a half-life that is 1- to 3-fold greater than the tumour residence time of the antibody is likely to be more therapeutically effective than a radionuclide with either a longer or a shorter half-life (Rao and Howell, 1993). Alternatively, a physical half-life similar to the biological half-life of the antibody in the tumour has also been suggested to be ideal (Schubiger and Smith, 1995).

The size of the tumour being treated, ie. solid tumours or micrometastatic disease, and the dynamics of the target antigen will play a role in selecting the appropriate radionuclide needed for RIT. These factors will determine which type of decay; beta (β); alpha (α); or electron capture; is most suitable for the particular treatment (Table 3). Iodine 131, which
Table 3. Radionuclides suitable for radioimmunotherapy.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Decay Mode</th>
<th>Physical $t_{1/2}$</th>
<th>Maximum Particulate Energy (% Abundance)</th>
<th>Maximum Range of Particulate Energy In Tissue (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{131}$I</td>
<td>Beta, gamma</td>
<td>8.0 days</td>
<td>807 keV (1)$^\dagger$, 606 keV (86)$^\dagger$, 336 keV (13)$^\dagger$</td>
<td>2.4</td>
</tr>
<tr>
<td>$^{67}$Cu</td>
<td>Beta, gamma</td>
<td>62 hours</td>
<td>577 keV (20)$^\dagger$, 484 keV (35)$^\dagger$, 395 keV (45)$^\dagger$</td>
<td>2.2</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>Beta</td>
<td>64 hours</td>
<td>2.29 MeV (100)$^\dagger$</td>
<td>11.9</td>
</tr>
<tr>
<td>$^{188}$Re</td>
<td>Beta</td>
<td>17 hours</td>
<td>2.13 MeV (100)$^\dagger$</td>
<td>11.1</td>
</tr>
<tr>
<td>$^{212}$Bi</td>
<td>Alpha, beta</td>
<td>1 hour</td>
<td>6.09 MeV (10)$^\ddagger$, 6.05 MeV (25)$^\ddagger$, 5.77 MeV (1)$^\ddagger$, 2.27 MeV (40)$^\ddagger$, 1.55 MeV (7)$^\ddagger$, 930 keV (5)$^\ddagger$, 670 keV (4)$^\ddagger$, 450 keV (5)$^\ddagger$, 85 keV (3)$^\ddagger$</td>
<td>0.09</td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>Alpha</td>
<td>7 hours</td>
<td>5.87 MeV (100)$^\ddagger$</td>
<td>0.09</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>Electron capture</td>
<td>60 days</td>
<td>35 keV (100)$^\ddagger$</td>
<td>0.02</td>
</tr>
</tbody>
</table>

$^\dagger$ Beta irradiation.

$^\ddagger$ Alpha irradiation.

Abbreviations: $^{131}$I, iodine 131; $^{67}$Cu, copper 67; $^{90}$Y, yttrium 90; $^{188}$Re, rhenium 188; $^{212}$Bi, bismuth 212; $^{211}$At, astatine 211; $^{125}$I, iodine 125.
decays by both gamma (γ)- and β-emissions is an attractive candidate for RIT. The γ-emissions do not deliver sufficient radiation doses to cause a tumouricidal effect, but instead, allow for the use of external scintigraphic imaging to determine the biodistribution of the radiolabeled antibody and to estimate the absorbed radiation doses from the β-emissions delivered to the tumour and normal tissues. However, the γ-emissions can deliver unnecessary radiation doses to normal tissues due to their long emission range.

Beta-emitters such as, yttrium 90 (90Y) or rhenium 188 (188Re), can deposit their particulate energy up to a maximum range of 11.9 and 11.1 mm in tissue, respectively, making them suitable for treating relatively large solid tumours. For tumours <5 mm, however, copper 67 (67Cu) and 131I, which possess maximum β-emission ranges of 2.2 and 1.4 mm, respectively, would be preferable over 90Y and 188Re because of their shorter range of emission. Using these radionuclides increases the likelihood that most of the energy released form the β-emissions is deposited within the tumour and not in neighbouring normal tissues. Mathematical modeling suggests that the optimum tumour diameters for maximizing the radiation dose delivered to the lesion (and potentially curability) are 28-42 mm, 23-32 mm, 2.6-5 mm, and 1.6-2.8 mm when 90Y, 188Re, 131I, and 67Cu, respectively, are utilized (O'Donoghue, 1996). The model also suggests that tumour curability could potentially decrease when the tumour diameter falls above or below these optimum ranges. If localized in a tumour with a diameter well below the calculated optimum range, 90Y would deposit most of its energy outside the lesion and in adjacent normal tissues. The model, however, assumes uniform tumour distribution of radionuclide and an equivalent phenotype, proliferation rate, and radiosensitivity amongst the tumour cells.

Because of their relatively long range, β-emitters such as, 131I, 90Y, or 67Cu, can produce a "cross-fire" effect (Nourigat et al., 1990). When bound to tumour cells, antibodies labeled with these radionuclides can irradiate nearby tumour cells that were not targeted by the radioantibody. Thus, heterogeneous antibody distribution in the tumour, arising from physiological barriers, i.e. the "binding-site barrier" phenomenon, poor vascularization, or
heterogeneous antigen distribution, may not necessarily hinder their tumouricidal effect. In contrast, using antibodies labeled with α-emitting radionuclides such as, bismuth 212 (\(^{212}\)Bi) and astatine 211 (\(^{211}\)At), may be ineffective when heterogeneous antibody distribution in the tumour occurs, due to their extremely short range of \(<90\ \mu m\), ie. \(<10\) cell diameters. More suitable tumour targets for α-emitters include, lymphoma, micrometastases, and compartmentally disseminated neoplasms such as, neoplastic meningitis (Zalutsky and Bigner, 1996). Radionuclides that decay by electron capture, ie. iodine 125 (\(^{125}\)I), have the shortest range of energy deposition, typically \(\leq20\ \mu m\). Thus, their ability for efficient tumour cell kill would require intracellular deposition, ie. internalization of the antigen-antibody complex. Due to their short emission ranges, \(^{212}\)Bi, \(^{211}\)At, and \(^{125}\)I lack a "cross-fire" effect but, on the other hand, can spare normal tissues from unnecessary irradiation.

Iodine 131 and \(^{90}\)Y are the two most commonly used radionuclides in experimental and clinical RIT studies. Iodine 131 has several advantages including, well-known radiochemistry, ready availability, and low cost (Wilder et al., 1996). On the other hand, the emission properties of \(^{90}\)Y enables it to deliver approximately 4.5-fold more radiation to a tumour than \(^{131}\)I, per mCi (Larson, 1991b). However, this also means that \(^{90}\)Y can deliver more radiation than \(^{131}\)I to normal tissues. In patients treated with \(^{131}\)I- and \(^{90}\)Y-mAbs, Stewart et al. (1988) observed that \(^{90}\)Y was 8-fold more toxic to bone marrow, per mCi, than \(^{131}\)I. These authors proposed that bone absorption of free \(^{90}\)Y can be the dominant source of bone marrow irradiation and toxicity from this radionuclide. On the other hand, Vriesendorp et al. (1996) suggest that circulating \(^{90}\)Y, labeled to a high molecular weight, slowly eliminated molecule, ie. an intact IgG mAb, is the dominant source of bone marrow irradiation and toxicity. Nevertheless, both \(^{90}\)Y and \(^{131}\)I have shown some success in clinical RIT. In a Phase I/II study by Lane et al. (1994), ten patients with colorectal cancer received anti-CEA \(^{131}\)I-ASB7 mAb (26-64 mCi), while nine patients received \(^{131}\)I-ASB7 F(ab')\(_2\) (82-148 mCi). One complete response was observed in the group that received \(^{131}\)I-ASB7 and one partial response was observed in the group treated with \(^{131}\)I-ASB7 F(ab')\(_2\). In both groups, however,
myelotoxicity was the limiting factor. In another Phase I trial, all of twenty-four colorectal cancer patients treated with $^{131}$I-CC49 (45-228 mCi) showed no major responses but, six patients did demonstrate stable disease for 4 weeks following initial treatment. Myelotoxicity was observed in eleven patients (Divgi et al., 1995). Knox et al. (1995a) treated eighteen patients with recurrent B-cell non-Hodgkin's lymphoma with 1-2 intravenous doses of anti-CD20 $^{90}$Y-2B8 mAb (13.5-50 mCi). Four patients demonstrated complete responses, nine patients showed partial responses but, myelotoxicity was observed.

Metabolism of Radionuclides

Following cellular internalization, radioiodinated antibodies rapidly undergo proteolysis and deiodination by degradative enzymes including, dehalogenases present in the lysosomes of tumour and certain normal tissue cells, ie. phagocytic cells such as, blood mononuclear cells and reticuloendothelial cells, resulting in the cleavage of radioiodine from the antibody (Wilder et al., 1996). Following lysosomal degradation, free radioiodine and small iodinated peptides are released from the cells and subsequently, are eliminated from the body by renal excretion, or in the case of free radioiodine, are secreted into the stomach or sequestered by the thyroid or salivary glands. On the other hand, cortical bone and liver are the major deposition sites for free $^{90}$Y (Durbin, 1960). The primary route of elimination of free $^{90}$Y is renal excretion, however, some of the $^{90}$Y deposited in the liver may be excreted through the gastrointestinal tract (Durbin, 1960). Compared to radioiodinated antibodies, $^{90}$Y-labeled antibodies display superior cellular retention when internalized and catabolized by tumour cells (Press et al., 1996) or normal blood mononuclear cells (Naruki et al., 1990). Radioiodine is conjugated to antibodies primarily by substitution onto tyrosine amino acids, whereas $^{90}$Y is chelated onto antibodies through chelating agents such as, diethylenetriaminepentaacetic acid (DTPA). Chelating agents such as, DTPA, can be conjugated onto the antibody via lysine residues (Hnatowich and McGann, 1987). Yttrium 90 ions circulating in blood can also be scavenged by transferrin (Moi et al., 1990).
Radiobiology

It has been suggested that to treat cancer successfully, a total radiation dose of 50-80 Gy must be delivered to the tumour (Bruland, 1995). The induction of double-strand breaks in the DNA is believed to be the mechanism by which RIT elicits tumour cell kill, rendering tumour cells incapable of division and growth (Cobb and Humm, 1986). Linear transfer energy represents the average energy locally imparted to a medium by a radiation particle when traversing 1 μm along its path (Hall, 1994). Since β-particles deposit their energy over a longer range (mm) compared to α-particles (μm), they are low linear transfer energy radionuclides, whereas α-emitters are high linear transfer energy radionuclides.

High linear transfer energy radiation is densely ionizing and is more efficient at producing DNA breaks compared to low linear transfer energy radiation. As a result, DNA breaks produced by α-particles are produced directly and are irreparable. In contrast, the DNA breaks produced by β-particles are done so indirectly, via highly reactive molecules, and are susceptible to repair (Hall, 1994). When β-emitting radionuclides are used, approximately 200 DNA double-strand breaks per cell are required to sterilize 99% of a tumour cell population, whereas only a few DNA breaks are required when α-emitters are used (Cobb and Humm, 1986). Similarly, only 3-6 impacts per nucleus by α-particles are required to sterilize up to 67% of tumour cells, but for β-particles, up to 400 times as many impacts are required (Humm, 1986).

In terms of dose rates, α-particles are cytotoxic at rates as low as 0.01 Gy/hour, whereas, even at dose rates of 0.1 Gy/hour, β-particles may not be tumouricidal (Fowler, 1990). Moreover, since DNA breaks produced by high linear transfer energy radiation are irreparable, α-emitters are cytotoxic even to hypoxic cells (Brown, 1986). For DNA breaks produced by β-emitters, however, hypoxia significantly reduces cytotoxicity (Hall, 1994). Since tumours are known to contain viable hypoxic cells, such radiobiological differences between α- and β-emitters can be significant.
The efficiency of RIT will depend on the total radiation dose delivered to the tumour, the dose rate, the duration of tumour irradiation, and the rate of cell proliferation. Andres et al. (1986) suggest that a minimal dose rate of 0.4 Gy/hour must be delivered to the tumour over a long enough period of time, so that all tumour cells are irradiated during the most radiosensitive M and G2 phases of their cell cycle. Typically, conventional XRT delivers dose rates of 60-180 Gy/hour to the tumour, while RIT provides dose rates of <0.6 Gy/hour (Hall, 1988). The dose rate delivered by XRT is constant and is delivered to a limited region of the body, i.e. the tumour and portions of adjacent tissues. In contrast, the dose rate delivered by RIT initially increases as the radioantibody accumulates in the tumour, then continuously decreases due to biological clearance and physical decay (O'Donoghue, 1994). Moreover, RIT delivers radiation to the entire body, not just the tumour and adjacent tissues. Labeling an antibody with a radionuclide such as, $^{90}$Y, which emits more energy per decay and has a shorter physical half-life (2.7 days), instead of $^{131}$I (half-life of 8 days), can produce a dose rate up to 10-fold greater (Wessels and Rogus, 1984).

Results from experimental tumour models have been quite variable in that the relative biological effectiveness of RIT has shown to be less (Buchsbaum et al., 1990), equal (Buras et al., 1990b), or superior (Wessels et al., 1989) to that of XRT. Possible explanations for the observed superior anti-tumour effect of RIT compared to dose-equivalent XRT, which is also known as the "inverse dose rate effect", include the following (Knox, 1995b): i) RIT targets a rapidly proliferating subpopulation of tumour cells that are primarily responsible for tumour doubling (Wessels et al., 1989); ii) reoxygenation of hypoxic tumour cells during the protracted course of RIT increases their radiosensitivity (Hall, 1994); iii) low dose rate irradiation induces apoptosis to a larger degree than high dose rate irradiation (Macklis et al., 1993); iv) the biological effects of some antibodies increase tumour blood flow and elicit an inflammatory response (Sands et al., 1988); and v) low dose rate irradiation leads to the accumulation of tumour cells in the radiosensitive G2 phase of the cell cycle (Knox et al., 1993).
To date, RIT of leukemias, lymphomas, and neuroblastomas has been most effective, whereas RIT of most solid tumours has been less promising (Kairemo, 1996). Radioimmunotherapy of lymphomas, in particular, has achieved the best clinical results and offers great prospects for success. As much as 50% of patients with non-Hodgkin's B-cell lymphoma have achieved complete responses from RIT (Wilder et al., 1996). Currently, leukemias and lymphomas appear to the most attractive candidates for RIT for several reasons: i) the cancers are relatively radioresponsive (Lybeert et al., 1987); ii) patients often have diminished HAMA responses due to supressed humoral immunity, which allows dose fractionation (DeNardo et al., 1995); and iii) leukemias and lymphomas, ie. hematological malignancies, are more readily accessible to antibodies than cells of solid, bulky tumours (DeNardo et al., 1988).

**Limitations of Radioimmunotherapy**

Response rates in clinical RIT studies (Table 2) have yet to match those obtained from animal studies (Table 1). Extrapolating the success from animal studies to clinical trials is difficult because of the inherent differences between animal models and humans. These include the following (Buchsbaum, 1995b; Knox, 1995b; Schubiger and Smith, 1995): i) differences between the pharmacokinetics and biodistribution, ie. tumour uptake in animal models is 10-35% of the injected dose per gram of tissue (% id/g), whereas <0.01% id/g is typically achieved in humans; ii) differences in the volume of distribution and the number of times the radiolabeled antibody circulates through the tumour vasculature; iii) the lack of HAMA responses in the murine model; iv) higher radiosensitivity of human bone marrow compared to murine bone marrow (Badger et al., 1985); and v) tumour xenografts in the animal model often have a better blood supply compared to tumours in patients (Goodwin, 1987).

Meanwhile, radioimmunoimaging has enjoyed greater clinical success, as is evident from the approval of two cancer imaging agents, Oncoscint CR/OV® and CEA-Scan®, by the US FDA for clinical use. Despite the tremendous amount of intense research dedicated to RIT
during the past 20 years, the major limitations of RIT have yet to be resolved, which include the following: i) only a minute fraction of the antibody dose localizes in solid tumours (<0.1%), resulting in the delivery of sub-therapeutic doses (<2 Gy); ii) the dose-limiting bone marrow toxicity, which has been observed in virtually all clinical studies; and iii) the HAMA response, which limits repeated courses of treatment as in dose fractionated RIT.

**Strategies to Improve Radioimmunotherapy**

In order to improve the clinical success of RIT, various approaches to improve antibody uptake in tumours, enhance tumour cytotoxicity, and reduce bone marrow toxicity and HAMA responses, have been investigated.

**Improving Tumour Uptake of Antibodies**

As described previously, using mAbs with greater affinity may increase their uptake and retention in tumours. Colcher et al. (1988) compared the tumour uptake of B72.3 mAb and its second generation mAb, CC49, in LS174T xenografts grown in athymic mice. CC49 mAb, which has a binding affinity constant (K_a) 6-fold greater than that of B72.3 demonstrated an approximately 2-fold higher uptake in the tumour. When studied in colorectal cancer patients, however, B72.3 and CC49 mAb did not show any significant differences in tumour uptake (Gallinger et al., 1993; Divgi et al., 1994). This discrepancy could be due to the presence of significant amounts of circulating target antigen, which favours antibody binding to the shedded antigen rather than the tumour-associated antigen on the surface of tumour cells or, to the overriding influence of the many barriers that tumour physiology imposes on antibody uptake.

Researchers have also attempted to increase tumour expression of the target antigen in order to increase tumour uptake of antibodies. Most of the research in this area has focused on the CEA and tumour-associated glycoprotein 72 (TAG-72) antigens. Experimental, as well as clinical evidence, have shown that interferon treatment can up-regulate the tumour surface
expression of CEA and TAG-72 antigens in a variety of cancer cells (Kantor et al., 1989; Greiner et al., 1990; Rosenblum et al., 1988; Murray et al., 1995). Elevation of specific mRNA transcripts following interferon treatment suggests that up-regulation is a result of new antigen synthesis (Kantor et al., 1989). The ability of interferon to enhance the therapeutic efficacy of RIT was demonstrated in an animal model (Greiner et al., 1993), in which administration of the cytokine to athymic mice bearing HT-29 colon cancer xenografts increased TAG-72 levels 10-fold and correspondingly, increased tumour localization of anti-TAG-72 $^{131}$I-CC49 mAb 3- to 4-fold. Mice that were administered interferon-$\gamma$ demonstrated a better therapeutic response to treatment with 300 $\mu$Ci $^{131}$I-CC49 compared to those not receiving interferon-$\gamma$. In a clinical trial, patients with late stage breast cancer were administered $\alpha$-interferon in combination with therapeutic doses of $^{131}$I-CC49 mAb (Murray et al., 1995). Some clinical responses were observed, however, the interferon and $^{131}$I-CC49 mAb combination may have also enhanced marrow toxicity.

Increasing intratumoural vascular permeability may also be effective in increasing tumour uptake of antibodies. Experimental evidence has shown that interleukin-2 (IL-2), administered in its free form (DeNardo et al., 1991) or conjugated to the targeting antibody (LeBerthon et al., 1991), can increase vascular permeability and antibody uptake in tumours. Increased vascular permeability may be a result of the interaction between activated lymphocytes and endothelial cells or through some direct effect of IL-2 and the endothelium (Allison et al., 1989). Low-dose XRT of the tumour has also been shown to be effective in increasing intratumoural vascular permeability in animal models and in patients. A total dose of 6-9 Gy from 2-3 courses of XRT ending one day prior to antibody administration achieved an approximately 3-fold improvement in tumour uptake in patients with hepatoma (Msirikale et al., 1987). Contrasting experimental evidence, however, suggest that this phenomenon may not occur with all tumours (Shrivastava, et al., 1989).

Other methods under investigation include, the use of local hyperthermia to reduce interstitial fluid pressure and enhance antibody uptake (Wilder et al., 1993; Leunig et al.,
administration of antibody "cocktails", which are composed of antibodies specific for different epitopes on the tumour cells (Blumenthal et al., 1991), and "pre-dosing" with unlabeled antibodies. "Pre-dosing" attempts to saturate Fc receptors on cells responsible for mAb catabolism and to form immune complexes with circulating antigen, prior to administering the radiolabeled antibody (Buchsbaum et al., 1992).

**Enhancing Tumour Cytotoxicity**

Animal models have shown that the proportion of hypoxic cells in the tumour increases as the tumour grows from microscopic to macroscopic size (Moulder and Rockwell, 1984). In some patients, tumour hypoxia has correlated with reduced efficacy of XRT (Höckel et al., 1993; Okunieff et al., 1993). Tumour hypoxia could also potentially diminish the effectiveness of RIT, particularly when low linear transfer energy radionuclides are employed. Improved tumour responses to radiation therapy have been reported following the administration of hyperbaric oxygen (Henk and Smith, 1977) or hypoxic cell sensitizers (Urtasun et al., 1976). Improved tumour responses to RIT following the use of pharmacological agents such as, tirapazamine (previously known as SR 4233), a direct-acting, first generation hypoxic cytotoxin, have been demonstrated in animal models (Wilder et al., 1993; 1994). Langmuir and Mendonca (1992) also demonstrated that RIT combined with SR 4233 treatment was at least 10-fold more cytotoxic than radiolabeled antibody alone. Experimental evidence suggests that under hypoxic conditions, tirapazamine becomes a free radical and is capable of inducing DNA strand breaks, leading to cell death during mitosis (Brown and Lemmon, 1990). In addition to increasing intratumoural vascular permeability, local hyperthermia can also be directly cytotoxic to certain radioresistant tumour cells, ie. hypoxic cells, acutely acidic cells, and cells in the S phase of the cell cycle (Hall, 1994; Sneed and Phillips, 1991). Both the temperature and duration of the treatment determines the degree of cell killing achieved. In a study by Wilder et al. (1993), local hyperthermia treatment for 1 hour and at a temperature of 43°C significantly enhanced the effectiveness of RIT of athymic mice bearing colorectal xenografts. In the same
study, a greater anti-tumour effect was observed when local hyperthermia and tirapazamine were combined with RIT.

Reducing HAMA

Attempts to abrogate HAMA have largely focused on the development and use of less immunogenic forms of mAbs such as, Fab, F(ab')2, and scFv fragments and humanized and chimeric intact mAbs (Figure 2). Although these derivatives lack large or entire portions of the murine constant regions, the possibility of HAMA responses directed against the variable regions exists (see: "Factors Influencing Tumour Uptake of Antibodies - Antibody Properties" and "Considerations for Radioimmunotherapy - Antibody Properties"). Based on the method for the production of murine mAbs established by Köhler and Milstein (1975), researchers have attempted to produce human antibodies (Glassy et al., 1987; Kozbor and Roder, 1981). These would also be much less immunogenic than intact murine mAbs, however, the instability of the hybridoma cells and low mAb yield remain the biggest limitations (Schubiger and Smith, 1995).

Pharmacological intervention with immunosuppressants such as, cyclosporin A, has also been attempted. In one clinical study, thirteen patients were given oral cyclosporin A two days prior to an initial administration of Fab or F(ab')2 fragments (Weiden et al., 1994). Six to 9 days later, patients then received either 186Re-labeled F(ab')2 or an intact mAb and were then given an additional course of oral cyclosporin A for up to 14 days. Of the patients who received cyclosporin, significant suppression of HAMA was observed in those with relatively higher cyclosporin levels. The cyclosporin-treated group also exhibited significant HAMA suppression compared to the patients who did not receive the immunosuppressant.

Rather than suppressing the HAMA response, attempts have been made to reduce the levels of circulating HAMA. Plasmapheresis, a procedure in which plasma is separated from cellular blood components has been shown to be effective in reducing HAMA levels in
patients. Patients in a RIT trial for the treatment of cutaneous T-cell lymphoma undergoing plasmapheresis had their HAMA titers reduced by 28-61% (Zimmer et al., 1988).

Reducing Bone Marrow Toxicity

Currently, the dose-limiting organ of RIT is the bone marrow. Granulocytopenia and thrombocytopenia, the two most common forms of myelotoxicity, reach nadir levels at approximately 4-6 weeks after the start of therapy. In most cases, the myelosupression is reversible, with white blood cell counts reaching normal levels 2-4 weeks later. Other side effects in other organ systems are rare and occur only after serious dose escalation (Vriesendorp et al., 1991) or with combination therapies (Wang et al., 1995). For example, after increasing the dose of $^{90}$Y-anti-ferritin mAb 4-fold to 4 mCi/kg, Vriesendorp et al. (1991) observed radiation hepatitis and massive ascites formation in beagle dogs, at 5 months following therapy. A similar dose escalation lead to the development of gastrointestinal toxicity in rats 5 days after treatment. In another study, Wang et al. (1995) observed radiation hepatitis and formation of ascites in beagle dogs 30 days after being treated with $^{90}$Y-ZCE025 mAb (0.5 mCi/kg) and external beam radiation of cobalt 60 to whole liver (30 Gy fractionated into 2 Gy/day at 5 days/week for 3 weeks). The dogs eventually developed terminal liver failure. Hematopoietic stem cell damage seems to be the most significant mechanism of bone marrow toxicity. Reducing or abrogating RIT-associated myelotoxicity is important because it could potentially permit higher amounts of radioactivity to be safely administered to patients, possibly improving the radiation dose delivered to the tumour.

Reducing the biological half-life of the radiolabeled antibody in the blood will reduce the radiation dose delivered to the bone marrow. Administration of secondary antibodies, ie. goat anti-mouse antibodies, as a clearing agent have demonstrated some success in reducing the bone marrow dose to patients (Begent et al., 1987). Secondary antibodies are directed against the radiolabeled antibodies and together form immune complexes in the circulation. The immune complexes are then quickly removed from blood in a manner analogous to that of the
immune complexes formed by HAMA. Another approach to enhance the clearance of the radiolabeled antibody involves the use of biotinylated anti-tumour antibodies, followed by administration of avidin or streptavidin as clearing agents. The very high affinity of the (strept)avidin/biotin interaction promotes the formation of immune complexes in the circulation, which are also quickly removed from blood. After administering streptavidin (at a 10-fold molar excess of the administered dose of radioantibody) to tumour-bearing mice 24 hours after administration of biotinylated $^{125}$I-anti-CEA mAb, Marshall et al. (1994) observed a 40% reduction in blood radioactivity levels. Interestingly, when the number of biotin molecules conjugated to the radiolabeled antibody was increased from four to nine biotins per mAb, blood radioactivity levels decreased even further, by 14-fold. By comparing the anti-tumour effect of radiolabeled antibody alone vs. that of radiolabeled antibody followed by secondary antibody, Blumenthal et al. (1989) observed that administration of a secondary antibody allowed a 2-fold increase in the administered dose of radioactivity, resulting in increased efficacy against the tumour.

Fab, F(ab')$_2$, and scFv fragments or MRUs offer an alternative approach to decrease the radiation dose to the bone marrow. As described previously, these derivatives possess a faster blood and normal tissue elimination compared to intact IgG mAbs as well as different properties with regard to tumour uptake and distribution (see: "Factors Influencing Tumour Uptake of Antibodies - Antibody Properties" and "Considerations for Radioimmunotherapy - Antibody Properties"). Despite promising results in experimental systems, so far no significant improvement in the therapeutic index over intact IgG mAbs has been observed in patients (Stigbrand, 1996; Lane et al., 1994; Mach et al., 1988). Further evaluation of mAb fragments will be required to assess their clinical utility in RIT.

Fractionated RIT is an approach that is less toxic to bone marrow. Meredith et al. (1992b) treated twelve colorectal cancer patients with either 2 x 14 mCi/m$^2$, 2 x 18 mCi/m$^2$, or 3 x 12 mCi/m$^2$ of $^{131}$I-chimeric B72.3 mAb. The fractionated dosing schedule reduced the degree of bone marrow toxicity, and produced a moderate increase in the therapeutic index.
However, despite using the less immunogenic chimeric form of the B72.3 mAb, HAMA responses were observed in 75% of the patients. In another study, dose fractionation of patients with B-cell malignancies resulted in ten complete or partial remissions in eighteen patients (DeNardo et al., 1990). The total radioactivity administered (300 mCi) was given in 30-60 mCi fractions and resulted in thrombocytopenia in only two patients. Although intact murine mAb was utilized, the frequency of HAMA was low, possibly reflecting the depressed immune function that is typical of patients with B-cell malignancies. Obviously, future clinical success of fractionated RIT will require reduction of HAMA responses. Other attempts to reduce bone marrow toxicity include relatively more complicated procedures such as autologous bone marrow transplantation (ABMT) (Press et al., 1989; 1993) and extracorporeal immunoadsorption (ECIA) (DeNardo et al., 1993). In ABMT, the patient's bone marrow is harvested prior to therapy and reinfused afterwards to restore the bone marrow stem cell population, whereas in ECIA, plasmapheresis techniques are used to remove excess circulating radioantibodies.

Adjunctive use of colony stimulating factors (CSFs) such as, interleukin-1 (IL-1), has been used to protect the bone marrow from the damaging effects of radiation. Interleukin-1 protects the hematopoietic stem cells from radiation (Gallicchio et al., 1989) and stimulates stem cell growth (Neta et al., 1987). Blumenthal et al. (1988) demonstrated in an animal model that IL-1 treatment 20 hours prior to the administration of \(^{131}I\)-labeled mAb could prevent bone marrow toxicity. In addition, IL-1 treatment 7 days following therapy was capable of reversing the bone marrow toxicity induced by the \(^{131}I\)-labeled antibody. Interleukin-1, however, is not capable of stimulating the growth of megakaryocytes and therefore, may have less of an effect on reducing thrombocytopenia.

Multi-step or pre-targeted RIT is another approach that can potentially reduce the radiation dose delivered to the bone marrow. Various pre-targeting protocols currently being investigated include, the complementary DNA oligonucleotide system (Kuijpers et al., 1993), the bispecific antibody-bivalent hapten system (Le Doussal et al., 1989), and the (strept)avidin-
biotin system (Paganelli et al., 1991a). In the latter two approaches, an antibody possessing binding affinity for both the target antigen and a small molecular weight effector molecule is administered to the patient. Following sufficient tumour uptake of the bifunctional antibody and clearance from the blood and normal tissues, the effector molecule, labeled with a therapeutic radionuclide, is then administered to deliver the radioactivity to the tumour. Excess effector molecule not localized in the tumour is then quickly eliminated from the body, ideally by renal excretion. Too long a delay between administration of the bifunctional antibody and effector molecule may result in sub-optimum tumour radiolocalization due to diffusion of the antibody from the tumour. The spatial distribution of the effector molecule in the tumour would be dictated by the intratumoural distribution of the antibody, and hence, appropriate radionuclides would have to be chosen on the basis of tumour size, antigen distribution, and the radiolabeling methods available. However, because of the small size of the effector molecule, diffusion from the vascular compartment into the tumour would be considerably faster than that of intact mAb. Therefore, the effector molecule could potentially deliver a higher initial dose rate to the tumour, assuming that similar radionuclides are used and absolute tumour uptake is not reduced due to rapid elimination of the effector molecule. Antigen internalization could have a profound effect on the effectiveness of pre-targeting protocols since it could reduce the number of binding sites available for the effector molecule on the surface of the tumour cells.

Non-specific irradiation of the bone marrow in pre-targeting approaches should be relatively small compared to that from RIT with radiolabeled intact mAbs or even F(ab')_2 fragments, since the effector molecule bearing the radionuclide is typically ≤1-2 kDa and exhibits a faster elimination from the blood. The ideal characteristics of an effector molecule include: i) low molecular weight and hydrophilicity; ii) rapid elimination from blood and renal excretion; and iii) little or no affinity for normal tissues. The half-life of certain effector molecules in the blood typically lasts for several hours, whereas that of intact murine mAb is typically several days long. Thus, pre-targeting is able to achieve high time-dependent tumour:normal tissue (T:NT) ratios more rapidly than conventional targeting with radiolabeled
mAbs. For example, administration of biotinylated AUAmAb followed by avidin 24 hours later (to clear excess antibody from blood) and then radiolabeled biotin, to tumour-bearing mice, achieved a tumour: blood (T:BLD) ratio of approximately 50:1 only 2 hours following injection of radiolabeled biotin. In contrast, administration of 131I-AUAmAb achieved a T:BLD ratio <1:1, more than 50-fold less than that achieved by the pre-targeting method (Paganelli et al., 1990a). Tumour: normal tissue ratios, which provide indices of radiolocalization, are calculated as the ratio between the % id/g in tumour and the % id/g in a normal tissue.

Pre-targeted RIT is an attractive approach to reduce bone marrow toxicity because: i) it does not require the use of pharmacological adjuvants; ie. IL-1; ii) the elimination half-life of radioactivity in the blood for pre-targeted RIT is short compared to that of radiolabeled mAbs and even F(ab')2 fragments; and iii) it avoids complex medical procedures such as, ABMT or ECIA, which could be associated with significant morbidity and mortality risks. Although there are much fewer experimental and clinical studies of pre-targeted RIT that have been conducted compared to conventional RIT, recent results are very encouraging (Axworthy et al., 1995; Cremonesi et al., 1997; Le Doussal et al., 1989). One promising approach for pre-targeted RIT is that involving the (strept)avidin-biotin system, which has previously been investigated by our laboratory in pre-targeted radioimmunoimaging of LS174T human colorectal xenografts grown subcutaneously in athymic mice (Alvarez-Diez et al., 1996).
PRE-TARGETING WITH THE (STREPT)AVIDIN-BIOTIN SYSTEM
There are three variations to the (strept)avidin-biotin system for pre-targeted RIT (Figure 3). These include: i) biotinylated mAb followed by radiolabeled (strept)avidin (2-step); ii) (strept)avidinylated mAb followed by radiolabeled biotin (2-step); and iii) biotinylated mAb followed by, (strept)avidin as a clearing agent, and radiolabeled biotin (3-step). These approaches exploit the very high binding affinity between the (strept)avidin and biotin molecules for tumor radiolocalization. Application of the (strept)avidin-biotin system to target specific antigens is not unique, for it is also widely used for *in vitro* applications in immunohistochemistry, enzyme linked immunosorbent assay, and molecular biology (Wilchek and Bayer, 1988).

**Properties of (Strept)avidin and Biotin**

Avidin molecules are a family of oligomeric proteins that are approximately 65 kDa in weight and are made up of four identical subunits, each weighing approximately 16 kDa. Each subunit can bind one molecule of biotin and thus, one molecule of avidin can bind up to four molecules of biotin. The binding sites, however, are fairly close to each other, which may offer some steric hindrance to the binding of large biotinylated molecules, in which case, the avidin protein may not necessarily behave tetravalently. The binding affinity between avidin and biotin is $10^{15}$ M$^{-1}$ (Green, 1963), approximately 1 million-fold greater than that of most antibody-antigen interactions. For practical purposes, the binding of biotin to avidin can be regarded as an irreversible process. The family of avidins include, proteins that are produced by amphibians, reptiles, and avians (Woolley and Longsworth, 1942; Korpela et al., 1981), as well as that produced by *Streptomyces avidinii*, which is known as streptavidin (Stapely et al., 1963). All avidins possess identical biotin-binding properties but, differ in physico-chemical properties. For example, avian avidins are very basic glycoproteins with an isoelectric point of approximately 10.5 (Woolley and Longsworth, 1942), thereby existing as a cationic species at physiological pH. Thus, *in vivo*, they are capable of associating with negatively charged compounds such as, mucopolysaccharides or nucleic acids. Streptavidin, on the other hand,
I. Biotinylated mAb and radiolabeled (strept)avidin (2-step)

II. (Strept)avidinylated mAb and radiolabeled biotin

III. Biotinylated mAb, (strept)avidin, and radiolabeled biotin

Figure 3. Schematic representation of the three variations to the (strept)avidin-biotin pretargeting system. I. Biotinylated mAb is first administered to the patients followed by radiolabeled (strept)avidin after sufficient tumour uptake and normal tissue elimination of antibody has occurred; II. (Strept)avidinylated mAb followed by radiolabeled biotin; III. Biotinylated mAb followed by unlabeled (strept)avidin following sufficient tumour uptake of the antibody. (Strept)avidin works as a clearing agent and provides binding sites for radiolabeled biotin in the tumour. After elimination of (strept)avidin-biotinylated mAb complexes, radiolabeled biotin is administered.
does not contain carbohydrate residues and is uncharged at physiological pH (Dittmer et al., 1989).

Biotin, formerly known as vitamin H, is a 244 Da molecule consisting of an aliphatic head and an aliphatic tail. The aliphatic head, a complex heterocycle, is believed to be the crucial part of the biotin molecule responsible for binding to avidin proteins (Green, 1975). No physiological compound other than biotin binds to avidins with any significant strength (Green, 1975). Biotin is an endogenous molecule, also found in animal and human tissues. In humans, the concentration of circulating biotin is estimated to be 0.5 ng/mL (Mock and Dubois, 1986) and in mice, is approximately 8-fold greater at 4 ng/mL (Rosebrough et al., 1991).

There is no evidence so far indicating that avidins may be toxic to humans, however, they do appear to be immunogenic, which is not an unexpected finding given their xenogeneic nature. Hnatowich et al. (1985a) reported that 8/10 patients administered streptavidinylated mAb displayed a human anti-streptavidin response (HASA), as well as a HAMA response. Paganelli et al. (1991b) also reported the development of HASA responses appearing 15-20 days following streptavidin administration in 7/20 patients in the study.

**Biotinylated mAb and Radiolabeled (Strept)avidin (2-step)**

Experimental evidence indicates that biotinylation of mAbs does not affect their biodistribution or elimination from blood in animals and patients (Sinitssyn et al., 1989; Paganelli et al., 1990a; Saga et al., 1994). The rate of tumour uptake and intratumoural distribution of biotinylated mAb should be similar to that of native mAb since biotinylation does not increase the size of the IgG molecule significantly. Saga et al. (1994) found the distribution of biotinylated D3 mAb in lung metastases to be similar to that of native D3 mAb. Biotinylation of mAbs can circumvent the loss of immunoreactivity due to radiolysis, a problem which has been reported for certain radiolabeled mAbs (Nikula et al., 1995). However, radiolysis of radiolabeled streptavidin is a possibility, that could adversely affect its
binding to biotinylated mAb. Similarly, radiolysis of radiolabeled biotin could also occur and affect its binding to streptavidinylated mAb. Paganelli et al. (1990a) reported no loss in immunoreactivity as a result of the biotinylation of AUA1 mAb. Mathematical modeling has projected that plasma clearance of streptavidin would be approximately 3-fold faster than that of intact mAb (Sung et al., 1994). Paganelli et al. (1992) reported that streptavidin, labeled with indium 111 (111In), had a β-phase half-life of 45 hours in ovarian cancer patients, following ip administration. However, when administered by iv injection, 111In-labeled streptavidin displayed a half-life in the blood of 16 hours (Paganelli et al., 1991a). Animal studies demonstrated that the biodistribution and pharmacokinetics of streptavidin differ substantially from that of avidin (Schechter et al., 1990; Rosebrough, 1993), which could have important implications in clinical pre-targeted RIT using biotinylated mAb and radiolabeled (strept)avidin. The elimination of streptavidin from blood is markedly slower than that of avidin, and unlike avidin, accumulates in the kidneys. Moreover, different preparations of streptavidin display markedly different biodistribution, at least in mice (Schechter et al., 1990). These authors observed that the truncated form of streptavidin, which is present in most commercial preparations, had an overall retention in blood and normal tissues, except kidney, that was approximately 3-fold lower than that of intact streptavidin. However, truncated streptavidin exhibited approximately an 8-fold increase in kidney localization compared to intact forms of the protein.

Tumour uptake of radioactivity using biotinylated mAb and radiolabeled streptavidin can be superior than that achieved using radiolabeled mAb. In a study by Paganelli et al. (1990a), tumour radiolocalization at 24 hours following ip administration of radiolabeled streptavidin to tumour-bearing mice, pre-targeted with biotinylated mAb, was 4-fold greater using the 2-step approach compared to that of radiolabeled mAb. In addition, blood levels of radioactivity were approximately 3-fold lower for radiolabeled streptavidin compared to radiolabeled antibody. However, increased liver uptake with the 2-step approach was observed due to the formation of immune complexes between circulating biotinylated mAb and
radiolabeled streptavidin. In an animal model, Saga et al. (1994) reported that the rate of tumour uptake of $^{125}$I-streptavidin in lung metastases, pre-targeted with biotinylated D3 mAb, was faster than that with conventional targeting with $^{131}$I-D3 mAb. However, it also appeared that the "binding site barrier" phenomenon may have prevented deep penetration of the streptavidin molecule in the tumour nodule. Given the very high binding affinity between streptavidin and biotin ($K_a=10^{15} \text{M}^{-1}$), intratumoural distribution of radiolabeled streptavidin may be more heterogeneous than that of the biotinylated mAb, with its binding limited to the biotinylated mAb adjacent to tumour vasculature. Mathematical modeling, in fact, predicts that the degree of intratumoural heterogeneity arising from the 2-step approach could be greater under some conditions (van Osdol et al., 1993).

(Strept)avidinylated mAb and Radiolabeled Biotin (2-step)

In this alternative 2-step approach, the radiolabeled effector molecule, biotin, is more rapidly eliminated from the blood than streptavidin. Paganelli et al. (1990b) determined, in patients, the half-life of biotin in blood to be approximately 3 minutes, when administered by iv injection. In the first 24 hours, up to 80% of the injected dose was excreted in the urine, while kidney uptake was only 3% of the injected dose. Similarly, Kalofinos et al. (1990), determined the $\alpha$-phase half-life of biotin, in serum, to be 2.4 minutes and the $\beta$-phase half-life to be 4.2 hours. Within 24 hours after administration, 71% of the injected activity was excreted in the urine. An advantage of using streptavidinylated mAbs and radiolabeled biotin is the potential amplification in the delivery of radioactivity to the tumour because of the tetravalent binding between (strept)avidin and biotin. Furthermore, unlike (strept)avidin, biotin does not appear to sequester in any normal tissues, at least when conjugated to DTPA (Rosebrough, 1993, Paganelli et al., 1991b). However, preliminary data suggest that the biodistribution of radiolabeled biotin depends on the physico-chemical properties of the chelating agent, ie. charge and lipophilicity (Paganelli et al., 1991a).
The presence of endogenous biotin may be a potential complication of using this 2-step approach since endogenous biotin may saturate the biotin binding sites of streptavidinylated mAb in the tumour. Rusckowski et al. (1997) demonstrated that endogenous biotin in mice can significantly interfere with binding of radiolabeled biotin to the tumour. In tumour-bearing mice pre-treated with streptavidin to reduce endogenous biotin, streptavidinylated mAb localized in tumour xenografts was capable of binding 95% of the administered radiolabeled biotin. In contrast, without pre-treatment, only 6% of radiolabeled biotin bound to streptavidinylated mAb. The presence of endogenous biotin, however, may not be as significant in humans since the concentration of circulating biotin is 8-fold less compared to that in mice (Rosebrough et al., 1991; Mock and Dubois, 1986). In a clinical study by Kalofonos et al. (1990), endogenous biotin did not saturate the biotin binding capacity of streptavidinylated mAb in 75% of the patients. Similar results were also found by Paganelli et al. (1991b). Administering higher doses of radiolabeled biotin may be an alternative strategy to circumvent any potential complications of endogenous biotin.

Mathematical modeling (van Osdol et al., 1993; Sung and van Osdol, 1995) predicted that, at similar doses, the intratumoural distribution of streptavidinylated mAb would be more heterogeneous than that of native mAb and biotinylated mAb because of its higher molecular weight. The addition of one streptavidin molecule to the mAb would increase its molecular weight from 150 kDa to approximately 215 kDa, whereas biotinylation does not significantly increase the molecular weight of the IgG molecule. Sung and van Osdol (1995) also predicted that the rate of tumour uptake of radiolabeled biotin would be considerably faster than that of native mAb and streptavidin because of its much smaller molecular weight. However, biotin may also be subject to the effects of the "binding site barrier" in the presence of pre-targeting with streptavidinylated mAb (van Osdol et al., 1993). Several investigators have reported no significantly large losses in mAb immunoreactivity as a result of streptavidinylation (Kalofonos et al., 1990; Ngai et al., 1995).
Axworthy et al. (1995) treated tumour-bearing mice with streptavidinylated mAb followed by, a clearing agent, and $^{90}$Y-DOTA-biotin, at doses ranging from 200-800 $\mu$Ci. More than 90% of the administered radioactivity was eliminated from the body within 24 hours following injection and no apparent myelotoxicity was observed. Complete tumour regression lasting for $\geq$200 days in 80% of mice bearing subcutaneous human breast tumours and in 100% of mice bearing subcutaneous human colorectal tumours was observed. The absorbed radiation doses delivered to the colorectal tumours were estimated to be $>150$ Gy/800 $\mu$Ci. In contrast, conventional RIT with $^{90}$Y-mAb demonstrated greater toxicity and lower therapeutic efficacy at equivalent doses up to 400 $\mu$Ci. At 400 $\mu$Ci, however, death in 100% of the mice was observed.

**Biotinylated mAb, (Strept)avidin, and Radiolabeled Biotin (3-step)**

The optimum time between administration of the bifunctional antibody and effector molecule will depend on how fast the antibody is eliminated from the blood and normal tissues. In the 3-step approach, elimination of biotinylated mAb from the blood can be encouraged by administering unlabeled (strept)avidin as a clearing agent. The (strept)avidin acts as a chase molecule, binding to circulating biotinylated mAb and forming immune complexes that are rapidly sequestered by the liver. In addition, (strept)avidin binds to biotinylated mAb localized in the tumour, thereby providing binding sites for the radiolabeled biotin to be administered in the third step. The 3-step approach has been successful in tumour imaging studies. In tumour-bearing mice, the 3-step approach achieved a T:BLD ratio of 50:1 at only 2 hours after injection of radiolabeled biotin, while that achieved by biotinylated mAb and radiolabeled streptavidin at 4 hours following injection of radiolabeled streptavidin was approximately 12-fold lower, ranging from 3.5:1 to 4:1 (Paganelli et al., 1990a). Moreover, the tumour:liver ratio was between 30:1 to 40:1 for the 3-step approach, whereas that of the 2-step approach was $<2:1$. Unlike with biotinylated mAb and radiolabeled streptavidin, very little liver uptake of radioactivity was observed in the 3-step approach perhaps because the sequestered immune
complexes were metabolized within the hepatocytes and unavailable for binding to radiolabeled biotin.

Cremonesi et al. (1997) used the 3-step approach to treat forty-five glioma patients. Patients were administered biotinylated mAb followed by 3 clearing agents, avidin, streptavidin, and biotinylated human serum albumin. In the third step, patients were administered $^{90}$Y-DOTA-biotin at a dose of 25-75 mCi/m² mixed with tracer amounts of $^{111}$In-DOTA-biotin to monitor the biodistribution of DOTA-biotin. Elimination of DOTA-biotin was rapid with 65% of administered radioactivity recovered in the urine within 24 hours. At 2 months post-therapy 56% of the patients demonstrated stable disease, 24% demonstrated progressive disease, and 20% had a complete response. However, at 12 months post-therapy, 84% of the patients demonstrated progressive disease and the percentage of patients demonstrating a complete response decreased to 16%. The absorbed radiation dose to the tumour was estimated to be $0.15 \pm 0.082$ Gy/mCi, while the dose to the bone marrow was $0.009 \pm 0.005$ Gy/mCi. Although a majority of the patients displayed hematological toxicity, the myelosupression observed was mild. Compared to the average radiation dose delivered to the tumour, the average doses delivered to other normal tissues were relatively low at $0.038 \pm 0.015, 0.015 \pm 0.013$, and $0.006 \pm 0.003$ Gy/mCi for kidneys, liver, and brain, respectively.

The 3-step approach possesses the advantages offered by both 2-step approaches. It utilizes the smaller sized biotinylated mAb, which theoretical models have predicted to have superior tumour distribution compared to streptavidinylated mAb. Secondly, the 3-step approach also utilizes biotin as the effector molecule, which has demonstrated to have a shorter half-life in blood, less cross reactivity with normal tissues, and a faster rate of tumour uptake, compared to streptavidin. However, the 3-step approach is more complex than 2-step pre-targeting because of the two, rather than one, time intervals to consider, as well as an additional reagent for which an appropriate dose must be determined.
Pre-targeting with Streptavidin-CC49 Monoclonal Antibody and DOTA-biotin

**CC49 Monoclonal Antibody**

A first generation mAb directed against TAG-72 antigen, B72.3, was generated by Colcher et al. (1981) by using a membrane-enriched fraction of a human breast carcinoma biopsy as the immunogen. The TAG-72 antigen is a high molecular weight mucin-like antigen expressed in >94% of human colorectal cancers and a majority of ovarian, breast, and non-small cell lung carcinomas (Johnson et al., 1986; Thor et al., 1986). Muraro et al. (1988) purified TAG-72 from the LS174T human colon cancer cell line and used the antigen to generate a series of twenty-eight second generation IgG mAbs, designated "CC" for colon cancer. The second generation mAb, CC49, demonstrated superior reactivity to TAG-72 as compared to B72.3. The $K_a$ of CC49 is $1.6 \times 10^{10} \text{ M}^{-1}$, whereas that of B72.3 is nearly 6-fold lower at $2.5 \times 10^9 \text{ M}^{-1}$. In the animal model, CC49 mAb has displayed superior tumour uptake and anti-tumour effects compared to B72.3, possibly as a result of its higher $K_a$ (Schlom et al., 1992; Colcher et al., 1988). In a phase I RIT study, Divgi et al. (1995) treated twenty-four colorectal cancer patients with $^{131}$I-CC49 mAb at a dose of 15-90 mCi/m². Ninety five percent of known lesions were visualized by $^{131}$I-CC49 mAb, thereby qualitatively demonstrating excellent tumour targeting by the radioantibody. Despite tumour localization, however, no major responses were observed. Nevertheless, six patients demonstrated stable disease for 4 weeks post-therapy and subsequently received a second dose of $^{131}$I-CC49 mAb. As a result, one patient had a minor response following the second treatment. Human anti-mouse antibody responses and dose-related hematological toxicity were observed. In a phase II RIT trial (Murray et al., 1994), fifteen patients were treated with 75 mCi/m² of $^{131}$I-CC49 mAb. Tumours were successfully imaged in all patients, however, no objective responses were seen. Absorbed radiation doses to the tumours were estimated to be between 0.19-66.7 Gy and HAMA and hematological toxicity resulted from treatment.
**Streptavidinylation of CC49 Monoclonal Antibody**

Streptavidin has been conjugated successfully to CC49 mAb in our lab (Ngai et al., 1995; Alvarez-Diez et al., 1996). Ngai et al. (1995) evaluated three different approaches to conjugate streptavidin to CC49 mAb. One approach involved conjugating the CC49 mAb with sulfosuccinimidyl-4-(N-maleimideomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) via lysine residues on the mAb. The maleimide-derivatized mAb was then reacted with chemically-thiolated streptavidin to generate the streptavidin-CC49 mAb conjugate. For the second conjugation approach, CC49 mAb was biotinylated and then reacted with streptavidin. Biotinylation was performed by reacting biotin LC-hydrazide with oxidized carbohydrate moieties on the Fc region of the mAb. In the third method, disulphide moieties on the CC49 mAb were reduced and then reacted with maleimide-derivatized streptavidin to produce the conjugate. Streptavidinylation in all three approaches did not largely affect the immunoreactivity of the CC49 mAb. The third approach provided the best conjugate yield at 95%, i.e. <5% free streptavidin, and produced a conjugate with a molecular weight of approximately 210 kDa, as determined by size-exclusion high pressure liquid chromatography (HPLC). This suggested that conjugation of only one streptavidin molecule (65 kDa) per CC49 mAb (150 kDa) was achieved. This conjugation procedure was subsequently used by Alvarez-Diez et al. (1996) in a 2-step pre-targeted radioimmunoimaging study, in which they successfully imaged subcutaneous LS174T human colorectal xenografts grown in athymic mice.

**DOTA-biotin**

1, 4, 7, 10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) is a synthetic macrocyclic chelator suitable for chelating radiometals such as, ⁹⁰Y, ¹¹¹In, and ⁶⁷Cu, and has been reported to form radiometal-chelator complexes more stable than those formed with other commonly used chelators such as, DTPA (Moi et al., 1990). Over an 18 day period in vitro, no measurable loss of yttrium 88 (⁸⁸Y) from DOTA was observed in serum under physiological
conditions. In contrast, a loss of 0.57%/day was observed when $^{88}$Y was chelated with 1-$p$-nitrobenzyl-DTPA. An even more dramatic loss of 20%/day was observed with the monobutylamide-DTPA derivative. Deshpande et al. (1990) also observed no measurable loss of $^{88}$Y in vitro from DOTA-conjugated Lym-1 mAb in serum under physiological conditions.

The NeoRx Corporation of Seattle, Washington has developed a DOTA-biotin molecule with a molecular weight of 900 Da for use in pre-targeting strategies (Su et al., 1995) (Figure 4). The DOTA-biotin compound labeled with $^{90}$Y has demonstrated rapid blood clearance in tumour-bearing mice pre-targeted with streptavidinylated mAb (Axworthy et al., 1995) (see: "Streptavidinylated mAb and Radiolabeled Biotin (2-step)") and in a clinical pre-targeted RIT study by Cremonesi et al. (1997) (see: "Biotinylated mAb, (Strept)avidin, and Radiolabeled Biotin (3-step)"). This same compound is also currently being used by our laboratory in evaluating streptavidin-CC49 and $^{90}$Y-DOTA-biotin for pre-targeted RIT of LS174T human colorectal xenografts grown subcutaneously in athymic mice (Domingo and Reilly, 1997).
Figure 4. Structure of 1, 4, 7, 10-tetraazaacycloodecan- N', N', N''-tetraacetic acid (DOTA) - bioin developed by the NeoRx Corporation.
RESEARCH OBJECTIVES
Statement of the Problem

Bone marrow toxicity is a major limitation of RIT because of the relatively long half-life of the radiolabeled antibody in blood. Moreover, bone marrow toxicity is dose-limiting, thereby making it difficult to deliver effective radiation doses to the tumour. A reduction in bone marrow toxicity can potentially enable higher doses of radioactivity to be safely administered to the patient and possibly, improve the radiation dose delivered to the tumour tissues.

Objectives of Research

The objective of my research is to evaluate streptavidin-CC49 and $^{90}$Y-DOTA-biotin for pre-targeted RIT of subcutaneous LS174T human colorectal xenografts grown in athymic mice. This will be accomplished by: i) evaluating the biodistribution of DOTA-biotin in BALB/c mice (Experiment I); ii) establishing a dosing regimen for streptavidin-CC49 and DOTA-biotin for pre-targeted RIT of athymic mice bearing LS174T colorectal xenografts (Experiment II); and iii) evaluating the therapeutic efficacy and toxicity of streptavidin-CC49 and $^{90}$Y-DOTA-biotin administered to athymic mice bearing LS174T colorectal xenografts (Experiment III).
EXPERIMENT I:

BIODISTRIBUTION OF DOTA-BIOTIN
Introduction

Our laboratory has successfully imaged subcutaneous human LS174T colorectal xenografts, grown in athymic mice, using a pre-targeting approach with streptavidin-CC49 and $^{111}$In-DTPA-biocytin (Alvarez-Diez et al., 1996). Rather than using DTPA-biocytin for our evaluation of pre-targeted RIT with streptavidin-CC49, we have chosen to utilize a DOTA-biotin compound developed by the NeoRx Corporation (Su et al., 1995). In vitro and in vivo studies have demonstrated that DOTA chelators can form a more stable complex with radioyttrium than can chelators such as, DTPA (Moi et al., 1990; Deshpande et al. 1990; DeNardo et al., 1994). Dissociation of $^{90}$Y from its chelator in vivo and subsequent accumulation of the free radionuclide in cortical bone is an important concern in RIT, because of the reported correlation between myelotoxicity and bone uptake of free $^{90}$Y ions (Stewart et al., 1988; 1990; Hnatowich et al., 1985b). Stewart et al. (1988) proposed that bone uptake of $^{90}$Y, can in fact, be a dominant source of bone marrow irradiation and toxicity. In a pre-clinical comparative toxicity study, a 220 μCi dose (LD$_{50}$) of $^{90}$Y-DTPA-BrE-3 mAb delivered a higher radiation dose to the bone marrow than did 308 μCi (LD$_{50}$) of $^{90}$Y-DOTA-BrE-3 mAb (DeNardo et al., 1994). These differences were attributed to a higher bone uptake of free $^{90}$Y, when administered in the form of $^{90}$Y-DTPA-BrE-3 mAb. Hence, in light of experimental and clinical evidence, DOTA-biotin was chosen as the effector molecule for our evaluation of pre-targeted RIT, in order to minimize the deposition of free $^{90}$Y in bone and any bone marrow toxicity that may arise as a result.

However, apart from forming stable radiometal-chelator complexes, the DOTA-biotin molecule should also display the following behaviour in vivo: i) rapid elimination from blood, primarily through renal excretion and ii) little or no affinity for normal tissues, in order to be a suitable effector molecule for pre-targeting strategies. The objective of this experiment, therefore, was to evaluate the biodistribution of the DOTA-biotin chelator in non-pre-targeted mice and verify that it displays those properties described above. Because of the similar chemistry between $^{111}$In and $^{90}$Y and the difficulty in obtaining accurate measurements of $^{90}$Y
radioactivity in tissues using gamma or beta-scintillation counters (Roselli et al., 1989). Others have used $^{111}\text{In}$ as a tracer for $^{90}\text{Y}$ in biodistribution studies (Leichner et al., 1997; Parker et al., 1990; Cremonesi et al., 1997). To monitor the biodistribution of DOTA-biotin, we labeled it with $^{111}\text{In}$ with the realization that, despite the similar chemistry between $^{111}\text{In}$ and $^{90}\text{Y}$, the stability of the $^{111}\text{In}$-DOTA complex may be slightly different than that of the $^{90}\text{Y}$-DOTA complex. Consequently, the biodistribution of DOTA-biotin, determined by the $^{111}\text{In}$ radioactivity measured in tissues, may be slightly different than that of DOTA-biotin when labeled with $^{90}\text{Y}$, due to differences in biodistribution between the two radionuclides (Durbin et al., 1960; Roselli et al., 1989).

**Methods**

*Preparation of $^{111}\text{In}$-DOTA-biotin* (see: Appendix IV)

DOTA-biotin was radiolabeled with $^{111}\text{In}$ chloride to a specific activity of 50 $\mu$Ci/$\mu$g. Indium $^{111}$-DOTA-biotin was diluted for injection with sterile 0.9% saline. The radiochemical purity of $^{111}\text{In}$-DOTA-biotin assessed by reversed-phase chromatography was 97%.

*Biodistribution of DOTA-biotin*

Fifteen four-week old female BALB/c mice (Charles River Laboratories, Boston, MA) were acclimatized in The Toronto General Hospital Animal Facility for two weeks upon arrival. Each mouse received 2 $\mu$g (100 $\mu$Ci) of $^{111}$In-DOTA-biotin by tail vein injection following sedation with subcutaneous injections (100 $\mu$L) of an anesthetic cocktail containing, 2.5%:(w/v) ketamine (Rogar/STB Inc., London, Ont.), 0.12%:(w/v) xylazine (Miles Canada Inc., Etobicoke, Ont.), and 0.025%:(w/v) acepromazine (Ayerst Laboratories, Montreal, Québec). Groups of 2-3 mice were sacrificed at 1, 2, 3, 6, and 12 hours following injection of $^{111}$In-DOTA-biotin and their tissues (blood, kidneys, liver, stomach, intestines, spleen, liver, heart, lungs, and right femur) collected. Tissue samples were weighed and counted for $^{111}$In radioactivity in a gamma well counter (Packard Model Auto-Gamma 5650, Downer's Grove, Ontario).
IL) using a window of 150-260 keV to include the 172 keV and 247 keV γ-photons of $^{111}$In. A sample of the $^{111}$In-DOTA-biotin injectate was also counted with the tissues to correct for physical decay. The biodistribution of DOTA-biotin was expressed as the % id/g of tissue, which was calculated as follows:

$$\left[ \frac{\text{activity}_{\text{tissue sample}}}{\text{activity}_{\text{injectate}}} \times \frac{\text{mass of tissue sample (g)}}{} \right] \times 100\%$$

**Results**

One mouse died from anesthetic overdose prior to injection with $^{111}$In-DOTA-biotin, and therefore, was removed from the study. Indium 111 radioactivity was rapidly eliminated from the blood with <2% id/g remaining at 1 hour post-injection (pi) of $^{111}$In-DOTA-biotin. At 2 and 12 hours pi, levels of radioactivity decreased by 5- and 100-fold, respectively (Figure 5). There was very little uptake of radioactivity in normal tissues such as, heart, lung, liver, spleen, and femur (Figure 6). Moreover, the radioactivity in these tissues was rapidly eliminated during the first 12 hours following injection of $^{111}$In-DOTA-biotin. From 1 hour pi to 12 hours pi, levels of radioactivity decreased by 40-, 70-, 60-, and 20-fold in the heart, lungs, liver, and femur, respectively. Kidneys had the highest levels of $^{111}$In radioactivity at 3.2% id/g at 1 hour pi, decreasing 8-fold to 0.4% id/g at 12 hours pi, which was still higher than other normal tissues, except intestines. In the intestines, there appeared to be some retention of $^{111}$In radioactivity with levels decreasing only slightly from 0.5% id/g at 1 hour pi to 0.4% id/g at 12 hours pi. The % id of $^{111}$In radioactivity per whole tissue was also determined and is presented in Table 4.

**Discussion**

We have chosen to use a DOTA-biotin compound developed by the NeoRx Corporation (Su et al., 1995) for our evaluation of pre-targeted RIT with streptavidin-CC49. DOTA chelators have been shown *in vitro* and *in vivo* to form a more stable complex with yttrium than chelators such as, DTPA (Moi et al., 1990; Deshpande et al., 1990; DeNardo et al., 1994).
Figure 5. Elimination of DOTA-biotin from blood. DOTA-biotin (2 μg) was labeled with $^{111}$In (100 μCi) and administered by tail vein injection to BALB/c mice. The mice were sacrificed at various time points following injection. Data points represent the mean % id/g of whole blood ± SD (n=3 except at 12 hours, n=2). Inset: data plotted on semi-log scale and fitted to dual phase exponential decay equation (GraphPad Prism software Version 2.0, GraphPad Software Inc., San Diego, CA).

Abbreviations: pi, post-injection; % id/g, % of injected dose per gram.
Figure 6. Tissue distribution of DOTA-biotin. DOTA-biotin (2 μg) was labeled with $^{111}$In (100 μCi) and administered by tail vein injection to BALB/c mice. The mice were sacrificed at various time points following injection. Bars represent the mean % id/g of tissue ± SD (n=3).

Abbreviations: pi, post-injection; % id/g, % of injected dose per gram; BLD, blood; KID, kidneys; HRT, heart; LUN, lungs; LIV, liver; SPL, spleen; STO, stomach; INT, intestines; FEM, femur.

$^a n=2$. 
Table 4. Tissue distribution of DOTA-biotin.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 (%)</th>
<th>2 (%)</th>
<th>3 (%)</th>
<th>6 (%)</th>
<th>12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5.44 ± 0.68</td>
<td>1.04 ± 0.35</td>
<td>0.29 ± 0.19</td>
<td>0.15 ± 0.07</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.88 ± 0.20</td>
<td>0.27 ± 0.13</td>
<td>0.13 ± 0.03</td>
<td>0.14 ± 0.004</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Heart</td>
<td>0.06 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.004 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.0002</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.30 ± 0.13</td>
<td>0.08 ± 0.06</td>
<td>0.01 ± 0.005</td>
<td>0.005 ± 0.002</td>
<td>0.002 ± 0.0001</td>
</tr>
<tr>
<td>Liver</td>
<td>0.87 ± 0.07</td>
<td>0.44 ± 0.23</td>
<td>0.20 ± 0.06</td>
<td>0.08 ± 0.03</td>
<td>0.03 ± 0.005</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.04 ± 0.008</td>
<td>0.01 ± 0.005</td>
<td>0.004 ± 0.0002</td>
<td>0.003 ± 0.0004</td>
<td>0.002 ± 0.0001</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.12 ± 0.009</td>
<td>0.08 ± 0.10</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.008</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>Intestines</td>
<td>1.03 ± 0.09</td>
<td>0.58 ± 0.16</td>
<td>0.51 ± 0.31</td>
<td>0.72 ± 0.26</td>
<td>1.03 ± 0.79</td>
</tr>
</tbody>
</table>

DOTA-biotin (2 μg) was labeled with $^{111}$In (100 μCi) and administered by tail vein injection to BALB/c mice. The mice were sacrificed at various time points following injection.

† n=3.
‡ n=2.
Minimizing the dissociation of $^{90}$Y \textit{in vivo} from its chelator is important in keeping the radiation dose delivered to the bone marrow and the risk of myelotoxicity as low as possible (DeNardo et al., 1994). In addition, the effector molecule should be rapidly eliminated from blood, primarily by renal excretion, and should demonstrate very little uptake in normal tissues. We evaluated the biodistribution of DOTA-biotin, labeled with $^{111}$In, in BALB/c mice at 1, 2, 3, 6, and 12 hours pi to verify that it exhibits those properties described above.

According to the biodistribution of $^{111}$In radioactivity, the DOTA-biotin chelator was rapidly eliminated from the blood (Figure 5) and had very little uptake in normal tissues (Figure 6). Chinol et al. (1997) evaluated the biodistribution of DOTA-biotin, labeled with $^{90}$Y, in tumour-bearing mice pre-targeted with biotinylated FO23C5 mAb and avidin. The DOTA-biotin compound used by Chinol et al., however, appears to have a chemical structure different to that of the DOTA-biotin used in this study. The molecular weights of both DOTA-biotin compounds appear to be similar but, the DOTA-biotin compound used by Chinol et al. is apparently susceptible to cleavage between the DOTA and biotin moieties, whereas the DOTA-biotin used in this study is not (Su et al., 1995). Nevertheless, results from both studies are comparable in that both DOTA-biotin compounds demonstrated relatively very little uptake in normal tissues. In our study, uptake of $^{111}$In radioactivity in normal tissues, including the femur, was $<0.3\%$ id/g at 3 hours pi, and in Chinol et al., normal tissue uptake of $^{90}$Y radioactivity was approximately $<0.2\%$ id/g at 4 hours pi. Chinol et al. (1997) reported that 90% of the injected activity was excreted into the urine at 4 hours pi of $^{90}$Y-DOTA-biotin. Although we did not measure for the presence of $^{111}$In excreted into the urine, the pattern of uptake and elimination of $^{111}$In in the kidneys suggest that DOTA-biotin was eliminated by renal excretion. In this study, there was some retention of radioactivity in the intestines, suggesting that the DOTA-biotin compound may have also been eliminated through hepatobiliary excretion. On the other hand, the retention of radioactivity may have represented elimination of free $^{111}$In, released from DOTA-biotin, through the gastrointestinal tract (Durbin, 1960).
This is not the first study to evaluate the biodistribution of the DOTA-biotin compound developed by the NeoRx Corporation. In a pre-clinical RIT study conducted by NeoRx (Axworthy et al., 1995), tumour-bearing mice were pre-targeted with streptavidin-mAb, administered a clearing agent, and then injected with $^{90}$Y-DOTA-biotin. More than 90% of the administered $^{90}$Y-DOTA-biotin was eliminated from the body within 24 hours pi. Moreover, no myelotoxicity was observed following treatment with 200-800 μCi of $^{90}$Y-DOTA-biotin. In a clinical study, Cremonesi et al. (1997) treated forty-five glioma patients with a 3-step pre-targeting protocol. Patients were administered biotinylated mAb followed by, avidin and streptavidin, and $^{90}$Y-DOTA-biotin. To monitor the biodistribution of DOTA-biotin, the final preparation was spiked with DOTA-biotin labeled with $^{111}$In. Approximately 65% of the administered $^{111}$In radioactivity was excreted into the urine and the radiation doses delivered to normal tissues from $^{90}$Y were low.

The results from our study must be interpreted with the realization that the biodistribution of DOTA-biotin, labeled with $^{111}$In, may be slightly different than that of DOTA-biotin labeled with $^{90}$Y. Roselli et al. (1989), however, demonstrated that substituting $^{111}$In as a tracer for $^{90}$Y can accurately predict the biodistribution of an $^{90}$Y-labeled species, if a suitable chelator is used. Using a chelator that forms radiometal-chelator complexes with both $^{111}$In and $^{90}$Y at equal stability, may lead to similar biodistribution when either radionuclide is used. The stability of $^{111}$In-DOTA and $^{90}$Y-DOTA complexes was not evaluated for this study. Nevertheless, this study demonstrated that DOTA-biotin is rapidly eliminated from blood, undergoes renal excretion, and has very little uptake in normal tissues, thereby indicating that, in most respects, it is suitable for pre-targeting strategies. Results from previous pre-clinical (Chinol et al., 1997; Axworthy et al., 1995) and clinical (Cremonesi et al., 1997) studies further support our conclusions.
EXPERIMENT II:

DEVELOPMENT OF A DOSING SCHEDULE
FOR PRE-TARGETED RADIOIMMUNOTHERAPY
WITH STREPTAVIDIN-CC49 MONOCLONAL ANTIBODY
AND $^{90}$Y-DOTA-BIOTIN
Introduction

Successful pre-targeted RIT requires that certain parameters be optimized, particularly those constituting the dosing schedule to be employed. These include: i) the dose of mAb conjugate to be administered; ii) the time interval(s) between the administration of the mAb conjugate, clearing agent (if applicable), and effector molecule; and iii) the dose of effector molecule to be administered, i.e., mass and radioactivity. Ideally, in a 2-step approach, enough mAb conjugate should be administered so that maximum tumour uptake of the antibody is achieved. Secondly, the time interval between the administration of the mAb conjugate and effector molecule should be one that allows for sufficient elimination of the antibody from the blood and normal tissues to occur, and in addition, one that maintains as high a level of antibody in the tumour as possible. Lastly, the administered dose of the effector molecule should saturate all of the available hapten binding sites in the tumour in order to maximize the radiation dose delivered to the tumour. Previously in our laboratory, Alvarez-Diez (1995) determined that a 250 μg dose of streptavidin-CC49 achieved higher absolute tumour uptake of antibody than did smaller doses of 50 and 100 μg. No dose higher than 250 μg was evaluated but, it was possible that greater tumour uptake of antibody may have been achieved at higher doses. Nevertheless, it was decided to continue using the 250 μg dose in our evaluation of pre-targeted RIT.

Determining the optimum parameters for pre-targeted RIT must be done experimentally, and therefore, the aim of this study was to develop a dosing schedule that will be subsequently employed in a pre-targeted RIT approach to treat athymic mice, bearing subcutaneous human LS174T colorectal xenografts, with streptavidin-CC49 and ⁹⁰Y-DOTA-biotin. The specific objectives were to determine the time interval and dose of effector molecule that meet the criteria described in the preceding paragraph. To determine these parameters, we performed dual label biodistribution studies in LS174T tumour-bearing mice, labeling streptavidin-CC49 with ¹²⁵I and DOTA-biotin with ¹¹¹In. Two different time intervals (40 and 72 hours) and four different doses of DOTA-biotin (1, 20, 40, and 150 μg) were evaluated.
Methods

Radiolabeling of Streptavidin with $^{125}\text{I}$

Iodine 125-streptavidin was prepared to a specific activity of 1 $\mu\text{Ci}/\mu\text{g}$ according to the Iodogen method (Fraker and Speck, 1978). Streptavidin (10 mg/mL) (Sigma Chemical Co., St. Louis, MO) was incubated with Na$^{125}\text{I}$ (Amersham, Oakville, Ont.) for 10 minutes at room temperature in a glass reaction vial coated with 20 $\mu\text{g}$ of 1, 3, 4, 6-tetrachloro-3a, 6a, diphenylglicourile (Iodogen) (Sigma Chemical Co.). At the completion of the radiolabeling procedure, $^{125}\text{I}$-streptavidin was purified from free $^{125}\text{I}$ on a Sephadex G-50 mini-column eluted with 0.9% saline (see: Appendix II for preparation of Sephadex G-50 mini-columns). The radiochemical purity of $^{125}\text{I}$-streptavidin was determined by paper chromatography using Whatman #1 chromatography paper. Radiochemical purity of $^{125}\text{I}$-streptavidin was $\geq$97%.

Preparation of ($^{125}\text{I}$-Streptavidin)-CC49 Monoclonal Antibody

CC49 mAb was purified from mouse ascites fluid and analyzed for purity by size-exclusion HPLC and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (see: Appendix I). CC49 mAb was then conjugated to $^{125}\text{I}$-streptavidin and analyzed for purity and molecular weight by size-exclusion HPLC and SDS-PAGE (see: Appendix II). The immunoreactive fraction of ($^{125}\text{I}$-streptavidin)-CC49 preparations ranged from 50-90% (see: Appendix III).

Preparation of $^{111}\text{In}$-DOTA-biotin (see: Appendix IV)

DOTA-biotin was labeled with $^{111}\text{In}$ chloride. The radiochemical purity of $^{111}\text{In}$-DOTA-biotin assessed by reversed-phase chromatography was $\geq$94%. Ninety one to 94% of $^{111}\text{In}$-DOTA-biotin bound to streptavidin in vitro. Indium 111-DOTA-biotin was diluted for injection with sterile 0.9% saline.
Biodistribution of Streptavidin-CC49 Monoclonal Antibody and DOTA-biotin

LS174T human colon cancer cells were injected subcutaneously in eleven six- to eight-week old female athymic mice and the tumour xenografts allowed to grow for 8-15 days, at which time tumour diameters ranged from 3-6 mm (see: Appendix V). In order to determine an appropriate time interval between the administration of streptavidin-CC49 and DOTA-biotin for the pre-targeted RIT study, the LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of (125I-streptavidin)-CC49 by ip injection followed by 1 μg (100 μCi) of 111In-DOTA-biotin, administered by tail vein injection, at either 40 or 72 hours pi (n=5-6 mice/time interval) of (125I-streptavidin)-CC49. Prior to receiving tail vein injections of 111In-DOTA-biotin, the mice were anesthetized as previously described in Experiment I. Six hours later, the mice were sacrificed and their tissues (tumour, blood, kidneys, liver, stomach, intestines, spleen, liver, heart, lungs, and right femur) collected. Two additional groups of LS174T tumour-bearing mice (n=6 mice/group) served as controls and received either 125I-streptavidin alone (50 μg; 50-60 μCi) by ip injection or 111In-DOTA-biotin alone (1 μg; 100 μCi) by tail vein injection. Mice receiving 125I-streptavidin and 111In-DOTA-biotin were sacrificed at 46 and 6 hours pi of radioactivity, respectively, and their tissues collected. To determine the appropriate dose of DOTA-biotin required for the pre-targeted RIT study, eighteen LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of (125I-streptavidin)-CC49 by ip injection. Results from the time interval study indicated that 40 hours was the appropriate time interval (see: Results and Discussion). Therefore, the mice received either 20, 40, or 150 μg (100-300 μCi) of 111In-DOTA-biotin by tail vein injection (n=6 mice/dose) 40 hours pi of (125I-streptavidin)-CC49. The mice were sacrificed at 6 hours pi of 111In-DOTA-biotin and their tissues collected.

All tissues were weighed and counted for 111In and 125I activity in a gamma well counter (Packard Model Auto-Gamma 5650) using windows of 150-260 keV and 15-80 keV, respectively. All measurements of 125I radioactivity were corrected for 111In spillover. Samples of the (125I-streptavidin)-CC49 and 111In-DOTA-biotin injectates were also counted with the tissues to correct for physical decay. The biodistribution of streptavidin-CC49 and
DOTA-biotin was expressed as the % id/g of tissue, calculated accordingly as previously described in Experiment I.

**Statistical Analysis**

Mean tissue uptake of radioactivity was compared between groups using the Student's unpaired t-test with a level of significance of p<0.05. Mean DOTA-biotin:streptavidin-CC49 ratios were compared between groups using one-way analysis of variance with a level of significance of p<0.05.

**Results**

Size-exclusion HPLC of CC49 mAb purified from mouse ascites fluid showed a single peak with retention times of approximately 8.0-8.5 minutes, which by extrapolation from a standard curve of molecular weight standards and their corresponding retention times, corresponded to a molecular weight of approximately 150 kDa (Figure 7). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified CC49 mAb showed only one protein band identified as having a molecular weight between 116-200 kDa (Lane B, Figure 8). Size-exclusion HPLC of streptavidin-CC49 revealed two peaks with retention times of approximately 7.2-7.8 minutes and 8.0-8.5 minutes, representing streptavidin-CC49 and unconjugated CC49 mAb, respectively (Figure 9). By extrapolation from the standard curve described above, the molecular weight of streptavidin-CC49 was estimated at 200-300 kDa. Size-exclusion HPLC did not detect the presence of any unconjugated streptavidin, which typically has a retention time of approximately 9.0 minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of streptavidin-CC49 preparations identified three protein bands (Lane C, Figure 8). The top two bands represent streptavidin-CC49, while the bottom band represents unconjugated CC49 mAb. The streptavidin-CC49 bands were identified as having a molecular weight >200 kDa, while the band representing unconjugated CC49 mAb was identified as having a molecular weight between 116-200 kDa. Sodium dodecyl sulpha-
Figure 7. Size-exclusion high pressure liquid chromatography analysis of CC49 mAb purified from mouse ascites fluid. The retention time of 8.24 minutes corresponds to a molecular weight of approximately 150 kDa.
Figure 8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of CC49 mAb and streptavidin-CC49. Samples were loaded onto a 4-20% Tris-glycine gel under non-reducing conditions and identified by Coomassie Blue R-250. Lanes A and E: molecular weight standards, myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and lysozyme (14 kDa); Lane B: CC49 mAb. Band corresponds to a molecular weight between 116-200 kDa; Lane C: streptavidin-CC49. Top two bands represent streptavidin-CC49 and correspond to a molecular weight >200 kDa. Lower band represents CC49 mAb. No unconjugated streptavidin was identified; Lane D: streptavidin. Protein dissociated into its subunits of 15 kDa.
Figure 9. Size-exclusion high pressure liquid chromatography analysis of streptavidin-CC49. The peaks with retention times of 7.60 and 8.47 minutes represent streptavidin-CC49 and unconjugated CC49 mAb, respectively. The streptavidin-CC49 corresponds to a molecular weight between 200-300 kDa and the unconjugated CC49 mAb corresponds to a molecular weight of approximately 150 kDa. No unconjugated streptavidin was detected, which has a retention time of approximately 9 minutes.
polyacrylamide gel electrophoresis confirmed those results obtained from size-exclusion HPLC in that, unconjugated streptavidin was not present in the streptavidin-CC49 preparations. By comparing the area under the peak representing unconjugated CC49 mAb on the HPLC chromatogram (Figure 9) to areas under the peak of known amounts of CC49 mAb, it was determined that the fraction of unconjugated CC49 mAb in the streptavidin-CC49 preparations was typically 50%.

The biodistribution of streptavidin-CC49, labeled with $^{125}$I, at two different time intervals is summarized in Table 5A. At both time intervals, the tumour, liver, and spleen demonstrated the highest uptake of radioactivity. Tumour uptake of radioactivity was $6.81 \pm 3.08\% \text{id/g}$ after the 40 hour time interval, whereas that after the 72 hour time interval was $4.26 \pm 1.22\% \text{id/g}$. These values were not significantly different, however, $^{125}$I radioactivity levels in the blood and liver after the 40 hour time interval were significantly higher compared to those after the 72 hour time interval. For the remaining normal tissues, levels of $^{125}$I radioactivity were not significantly different after either time interval. For the streptavidin control, the biodistribution was evaluated at 46 hours pi in order to be comparable to the biodistribution of streptavidin-CC49 after the 40 hour time interval. Uptake of streptavidin-CC49 in the tumour, liver, and spleen was approximately 3-, 7-, and 4-fold higher, respectively, compared to that of the streptavidin control. Kidney uptake of streptavidin, however, was extremely high, approximately 35-fold higher than that of streptavidin-CC49. The % id of $^{125}$I radioactivity per whole tissue was also determined and is summarized in Table 5B.

The T:NT ratios for streptavidin-CC49, at two different time intervals, are presented in Table 6. For both time intervals, the T:NT ratios were $\geq 1:1$, with exception of the tumour:liver ratios. The T:NT ratios for the 40 hour time interval ranged from 0.5:1 (tumour:liver) to 13.1:1 (tumour:intestines) and those for the 72 hour time interval, ranged from 0.4:1 (tumour:liver) to 11.7:1 (tumour:intestines). For the streptavidin control, the T:NT ratios were also $\geq 1:1$, with
Table 5A. Biodistribution of streptavidin-CC49 using a 40 or 72 hour time interval between administration of (125I-streptavidin)-CC49 and 111In-DOTA-biotin.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Streptavidin-CC49† 46 hours pi</th>
<th>Streptavidin-CC49‡ 78 hours pi</th>
<th>Streptavidin (control) 46 hours pi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumour</strong></td>
<td>6.81 ± 3.08c</td>
<td>4.26 ± 1.22</td>
<td>2.19 ± 0.43f</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>1.82 ± 0.75a</td>
<td>0.74 ± 0.18b</td>
<td>0.65 ± 0.15</td>
</tr>
<tr>
<td><strong>Kidneys</strong></td>
<td>1.10 ± 0.25g</td>
<td>0.92 ± 0.21</td>
<td>38.96 ± 7.80h</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td>0.56 ± 0.29</td>
<td>0.43 ± 0.10</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td><strong>Lungs</strong></td>
<td>0.91 ± 0.06</td>
<td>0.49 ± 0.07</td>
<td>0.87 ± 0.17</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>15.99 ± 2.72c,i</td>
<td>10.37 ± 1.63d</td>
<td>2.17 ± 0.46i</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>4.90 ± 1.07k</td>
<td>4.13 ± 0.33</td>
<td>1.24 ± 0.14i</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>1.46 ± 0.51</td>
<td>1.07 ± 0.54</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td><strong>Intestines</strong></td>
<td>0.60 ± 0.16</td>
<td>0.39 ± 0.12</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td><strong>Femur</strong></td>
<td>1.08 ± 0.28</td>
<td>0.78 ± 0.32</td>
<td>0.63 ± 0.10</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of (125I-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 μg (100 μCi) of 111In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (pi) of (125I-streptavidin)-CC49. Mice were sacrificed at 6 hours pi of 111In-DOTA-biotin. Control mice were administered 50 μg (50-60 μCi) of 125I-streptavidin by ip injection and sacrificed at 46 hours pi.

† Mean of 5-6 mice.
‡ Biodistribution is at 6 hours pi of 111In-DOTA-biotin.
a-b Significantly different at p=0.0069; c-d p=0.0021; c-f p=0.0051; g-h p<0.0001; i-j p<0.0001; k-l p<0.0001.
Table 5B. Biodistribution of streptavidin-CC49 using a 40 or 72 hour time interval between administration of $^{125}$-streptavidin)-CC49 and $^{111}$In-DOTA-biotin.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Streptavidin-CC49&lt;sup&gt;‡&lt;/sup&gt; &lt;br&gt;46 hours &lt;i&gt;pi&lt;/i&gt;</th>
<th>Streptavidin-CC49&lt;sup&gt;‡&lt;/sup&gt; &lt;br&gt;78 hours &lt;i&gt;pi&lt;/i&gt;</th>
<th>Streptavidin (control) &lt;br&gt;46 hours &lt;i&gt;pi&lt;/i&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour</td>
<td>0.84 ± 0.57</td>
<td>0.14 ± 0.09</td>
<td>0.11 ± 0.08</td>
</tr>
<tr>
<td>Blood</td>
<td>5.47 ± 2.24</td>
<td>2.21 ± 0.55</td>
<td>1.95 ± 0.46</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.39 ± 0.13</td>
<td>0.33 ± 0.08</td>
<td>1.71 ± 0.25</td>
</tr>
<tr>
<td>Heart</td>
<td>0.10 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.18 ± 0.04</td>
<td>0.08 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>12.80 ± 2.18</td>
<td>4.84 ± 0.62</td>
<td>3.26 ± 0.69</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.57 ± 0.10</td>
<td>0.45 ± 0.06</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.90 ± 0.15</td>
<td>0.80 ± 0.22</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Intestines</td>
<td>1.47 ± 0.44</td>
<td>1.45 ± 0.39</td>
<td>3.11 ± 4.15</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of $^{125}$-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 μg (100 μCi) of $^{111}$In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (<i>pi</i>) of $^{125}$-streptavidin)-CC49. Mice were sacrificed at 6 hours <i>pi</i> of $^{111}$In-DOTA-biotin. Control mice were administered 50 μg (50-60 μCi) of $^{125}$-streptavidin by ip injection and sacrificed at 46 hours <i>pi</i>.

† Mean of 5-6 mice.

‡ Biodistribution is at 6 hours <i>pi</i> of $^{111}$In-DOTA-biotin.
Table 6. Tumour: normal tissue ratios of streptavidin-CC49 using a 40 or 72 hour time interval between administration of $^{125}\text{I}$-streptavidin)-CC49 and $^{111}$In-DOTA-biotin.

<table>
<thead>
<tr>
<th>Normal Tissue</th>
<th>Streptavidin-CC49(^\ddagger) 46 hours pi</th>
<th>Streptavidin-CC49(^\ddagger) 78 hours pi</th>
<th>Streptavidin (control) 46 hours pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4.3 ± 2.3</td>
<td>6.0 ± 2.0</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Kidneys</td>
<td>6.5 ± 3.2</td>
<td>4.9 ± 2.3</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>10.6 ± 5.6</td>
<td>10.3 ± 3.5</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>Lungs</td>
<td>7.5 ± 3.2</td>
<td>8.9 ± 3.1</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Liver</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.5 ± 1.0</td>
<td>1.0 ± 0.2</td>
<td>4.6 ± 7.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>6.4 ± 6.8</td>
<td>4.8 ± 2.7</td>
<td>5.4 ± 1.6</td>
</tr>
<tr>
<td>Intestines</td>
<td>13.1 ± 9.1</td>
<td>11.7 ± 4.6</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Femur</td>
<td>6.6 ± 2.9</td>
<td>6.4 ± 3.2</td>
<td>3.5 ± 0.7</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of $^{125}\text{I}$-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 μg (100 μCi) of $^{111}$In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (pi) of $^{125}\text{I}$-streptavidin)-CC49. Mice were sacrificed at 6 hours pi of $^{111}$In-DOTA-biotin. Control mice were administered 50 μg (50-60 μCi) of $^{125}\text{I}$-streptavidin by ip injection and sacrificed at 46 hours pi.

\(^\ddagger\) Calculated as the ratio of the % injected dose/g of tumour and the % injected dose/g of normal tissue.

\(^\ddagger\) Mean of 5-6 mice.

\(^\ddagger\) Tumour: normal tissue ratios based on biodistribution at 6 hours pi of $^{111}$In-DOTA-biotin.
exception of the tumour:kidneys ratio. The T:NT ratios ranged from 0.1:1 (tumour:kidneys) to 5.8:1 (tumour:intestines) (Table 6).

The biodistribution of DOTA-biotin, labeled with $^{111}$In, at different time intervals is presented in Table 7A. There was no significant difference in the tissue uptake of radioactivity following administration of DOTA-biotin at either time interval. The % id/g of $^{111}$In radioactivity remaining in the blood was also not significantly different between the two time intervals. However, pre-targeting with the 40 hour time interval resulted in a 5- and 26-fold higher concentration of $^{111}$In radioactivity in the tumour and blood, respectively, compared to the DOTA-biotin control. The % id of $^{111}$In radioactivity per whole tissue was also calculated and is presented in Table 7B.

The T:NT ratios for DOTA-biotin, at two different time intervals, are presented in Table 8. For both time intervals, the T:NT ratios were >1:1, with exception of the tumour:kidneys ratios. For the 40 hour time interval, the T:NT ratios ranged from 0.5:1 (tumour:kidneys) to 19.3:1 (tumour:stomach) and for the 72 hour time interval, the ratios ranged from 0.4:1 (tumour:kidneys) to 17.6:1 (tumour:stomach). For the DOTA-biotin control, the T:NT ratios ranged from 0.1:1 (tumour:kidneys) to 14:1 (T:BLD) (Table 8).

Forty hours was chosen as the time interval to be employed for the pre-targeted RIT study, and hence, was used to evaluate increasing DOTA-biotin doses of 1, 20, 40, and 150 μg (see: Discussion). As the administered dose of DOTA-biotin was increased, the absolute tumour uptake of DOTA-biotin, expressed as pmols/g of tumour, also increased (see: Appendix VI for calculations). At doses of 1, 20, 40, and 150 μg, the absolute tumour uptake of DOTA-biotin was $7.05 \pm 2.68$, $38.47 \pm 5.84$, $123.78 \pm 96.13$, and $240.88 \pm 59.34$ pmols/g of tumour, respectively. At a constant dose of 250 μg, streptavidin-CC49 achieved an absolute tumour uptake of $34.37 \pm 26.78$ pmols/g of tumour (see: Appendix VI). Hence, administration of DOTA-biotin at doses of 1, 20, 40, and 150 μg corresponded to DOTA-biotin:streptavidin-CC49 ratios per g of tumour of $0.19 \pm 0.06$, $1.69 \pm 0.21$, $2.86 \pm 0.76$, and $9.28 \pm 3.39$ (see: Appendix VI) (Figure 10A). However, after normalizing for blood
Table 7A. Biodistribution of DOTA-biotin following pre-targeting with streptavidin-CC49 using a 40 or 72 hour time interval.

![Table](https://via.placeholder.com/150)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(+) Pre-targeting 40 hour time interval</th>
<th>(+) Pre-targeting 72 hour time interval</th>
<th>(-) Pre-targeting (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour</td>
<td>0.64 ± 0.24a</td>
<td>0.90 ± 0.75</td>
<td>0.14 ± 0.07b</td>
</tr>
<tr>
<td>Blood</td>
<td>0.26 ± 0.12c</td>
<td>0.27 ± 0.14</td>
<td>0.01 ± 0.003d</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1.26 ± 0.53</td>
<td>2.10 ± 1.33</td>
<td>1.08 ± 0.17</td>
</tr>
<tr>
<td>Heart</td>
<td>0.08 ± 0.04</td>
<td>0.07 ± 0.04</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.13 ± 0.04</td>
<td>0.12 ± 0.06</td>
<td>0.02 ± 0.005</td>
</tr>
<tr>
<td>Liver</td>
<td>0.36 ± 0.05</td>
<td>0.34 ± 0.15</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.11 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.03 ± 0.009</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.06 ± 0.04</td>
<td>0.06 ± 0.04</td>
<td>0.17 ± 0.15</td>
</tr>
<tr>
<td>Intestines</td>
<td>0.38 ± 0.16</td>
<td>0.25 ± 0.07</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>Femur</td>
<td>0.07 ± 0.03</td>
<td>0.17 ± 0.16</td>
<td>0.02 ± 0.005</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of (125I-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 μg (100 μCi) of 111In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (pi) of (125I-streptavidin)-CC49. Control mice were administered 1 μg (100 μCi) of 111In-DOTA-biotin by tail vein injection. All mice were sacrificed at 6 hours pi of 111In-DOTA-biotin.

† Mean of 5-6 mice.
‡ Biodistribution is at 6 hours pi of 111In-DOTA-biotin.

a-b Significantly different at p=0.0007; c-d p=0.0005.
Table 7B. Biodistribution of DOTA-biotin following pre-targeting with streptavidin-CC49 using a 40 or 72 hour time interval.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(+) Pre-targeting</th>
<th>(-) Pre-targeting (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 hour time interval</td>
<td>72 hour time interval</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.08 ± 0.05</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>Blood</td>
<td>0.88 ± 0.41</td>
<td>0.81 ± 0.42</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.39 ± 0.14</td>
<td>0.72 ± 0.41</td>
</tr>
<tr>
<td>Heart</td>
<td>0.01 ± 0.004</td>
<td>0.009 ± 0.005</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.009</td>
</tr>
<tr>
<td>Liver</td>
<td>0.46 ± 0.09</td>
<td>0.46 ± 0.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.01 ± 0.003</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.08 ± 0.10</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Intestines</td>
<td>1.28 ± 0.77</td>
<td>0.92 ± 0.27</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 µg (50-60 µCi) of $^{125}$I-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 µg (100 µCi) of $^{111}$In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (pi) of $^{125}$I-streptavidin)-CC49. Control mice were administered 1 µg (100 µCi) of $^{111}$In-DOTA-biotin by tail vein injection. All mice were sacrificed at 6 hours pi of $^{111}$In-DOTA-biotin.

† Mean of 5-6 mice.

‡ Biodistribution is at 6 hours pi of $^{111}$In-DOTA-biotin.
Table 8. Tumour:normal tissue ratios of DOTA-biotin following pre-targeting with streptavidin-CC49 using a 40 or 72 hour time interval.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(+) Pre-targeting(^{+}) 40 hour time interval</th>
<th>(+) Pre-targeting(^{+}) 72 hour time interval</th>
<th>(-) Pre-targeting (control)(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.7 ± 1.1</td>
<td>3.2 ± 1.5</td>
<td>14.0 ± 15.8</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>10.4 ± 6.1</td>
<td>12.1 ± 6.5</td>
<td>11.2 ± 6.4</td>
</tr>
<tr>
<td>Lungs</td>
<td>5.6 ± 3.3</td>
<td>7.2 ± 2.6</td>
<td>6.4 ± 4.6</td>
</tr>
<tr>
<td>Liver</td>
<td>1.8 ± 0.9</td>
<td>2.4 ± 1.1</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.2 ± 3.0</td>
<td>8.0 ± 4.2</td>
<td>4.8 ± 3.9</td>
</tr>
<tr>
<td>Stomach</td>
<td>19.3 ± 23.8</td>
<td>17.6 ± 10.7</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>Intestines</td>
<td>2.0 ± 1.3</td>
<td>3.6 ± 2.6</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Femur</td>
<td>9.8 ± 4.2</td>
<td>9.1 ± 7.3</td>
<td>7.8 ± 5.5</td>
</tr>
</tbody>
</table>

LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of \(^{125}\)I-streptavidin)-CC49 by intraperitoneal (ip) injection followed by 1 μg (100 μCi) of \(^{111}\)In-DOTA-biotin, administered by tail vein injection, at 40 or 72 hours post-injection (pi) of \(^{125}\)I-streptavidin)-CC49. Control mice were administered 1 μg (100 μCi) of \(^{111}\)In-DOTA-biotin by tail vein injection. All mice were sacrificed at 6 hours pi of \(^{111}\)In-DOTA-biotin.

\(^{+}\) Calculated as the ratio of the % injected dose/g of tumour and the % injected dose/g of normal tissue.

\(^{\dagger}\) Mean of 5-6 mice.

\(^{\ddagger}\) Tumour:normal tissue ratios based on biodistribution at 6 hours pi of \(^{111}\)In-DOTA-biotin.
Figure 10. Tumour uptake of DOTA-biotin at increasing doses. LS174T tumour-bearing mice were administered 250 μg (50-60 μCi) of ([125I]-streptavidin)-CC49 by intraperitoneal injection and at 40 hours post-injection (pi) were administered 1, 20, 40, and 150 μg (100-300 μCi) of [111In]-DOTA-biotin by tail vein injection. The mice were sacrificed at 6 hours pi of [111In]-DOTA-biotin. Data points represent the mean DOTA-biotin:streptavidin-CC49 ratio per g of tumour ± SD (n=6 except at the 1 μg dose, n=5). (A) Ratios are not normalized to [111In] radioactivity in blood; (B) Ratios are normalized to [111In] radioactivity in blood. (see: Appendix VI for calculation of ratios).
radioactivity, the DOTA-biotin:streptavidin-CC49 ratios per g of tumour were 0.70 ± 0.19, 0.99 ± 0.22, 1.33 ± 0.41, and 1.94 ± 0.46 at DOTA-biotin doses of 1, 20, 40, and 150 µg, respectively (see: Appendix VI) (Figure 10B). Figure 10A shows that without normalization for blood radioactivity, the DOTA-biotin:streptavidin-CC49 ratios per g of tumour increased almost linearly with increasing doses of DOTA-biotin. In contrast, Figure 10B demonstrates that with normalization for blood radioactivity, the ratios reached a plateau as the DOTA-biotin dose was increased from 40 to 150 µg.

Discussion

To increase the likelihood of successful pre-targeted RIT, an appropriate dosing schedule must be employed. For 2-step approaches, sufficient amounts of mAb conjugate should be administered to saturate the available binding sites in the tumour. Secondly, the time interval should be one that allows for sufficient elimination of the antibody from the blood and normal tissues to occur, and in addition, one that maintains as high a level of antibody in the tumour as possible. Lastly, the administered dose of effector molecule should be one that saturates the available hapten binding sites in the tumour to deliver as high a radiation dose to the tumour as possible. Dual label biodistribution studies were performed in LS174T tumour-bearing mice to experimentally determine a time interval and DOTA-biotin dose that meet these criteria. Two different time intervals (40 and 72 hours) and four different doses of DOTA-biotin (1, 20, 40, and 150 µg) were evaluated.

The T:NT ratios for streptavidin-CC49, at both time intervals, were ≥1:1, with exception of the tumour:liver ratios (Table 6), suggesting that tumour localization with streptavidin-CC49 was achieved. The low tumour:liver (≤0.5:1) and tumour:spleen ratios (≤1.5:1), at both time intervals, are a result of the high liver and spleen uptake of radioactivity. The liver and spleen are reported to be responsible for the catabolism of antibodies and other protein macromolecules (Wagle et al., 1978), most likely explaining the relatively high uptake of radioactivity demonstrated by these tissues. Liver uptake of radioactivity, at both time
intervals, was 10.37-15.99 %id/g and spleen uptake was 4.13-4.90% id/g (Table 5). For the streptavidin control, however, liver uptake of radioactivity was only 2.17 ± 0.46% id/g and spleen uptake was only 1.24 ± 0.14% id/g. The apparent higher uptake of streptavidin-CC49 in the liver and spleen may be due to Fc receptor-mediated uptake of the mAb conjugate (via the Fc regions of the CC49 mAb) by the Kupffer cells, hepatocytes, and other Fc receptor-bearing cells residing in these tissues. With exception of the tumor:kidneys ratio, the T:NT ratios for the streptavidin control were ≥1:1, suggesting that the protein achieved some non-specific tumor localization (Table 6). Similar non-specific tumor uptake of streptavidin has also been reported by others. Hnatowich et al. (1993) successfully imaged LS174T colorectal xenografts by administering unconjugated streptavidin followed by $^{111}$In-EDTA-biotin. Zhang et al. (1997) administered $^{131}$I-avidin alone to treat SH1N3 human ovarian cancer xenografts, resulting in a significant reduction in tumor weight. Thomas et al. (1989) suggested that tumor capillaries may be up to 10-fold more permeable than capillaries of normal tissues, possibly explaining the non-specific tumor uptake of streptavidin observed in this and other studies. Very high kidney uptake of radioactivity (38.96 ± 7.80% id/g) following administration of the streptavidin control was observed in this study (Table 5). Similar observations in mice biodistribution studies have also been reported by others. Hnatowich et al. (1993) observed a kidney uptake of streptavidin of 25% id/g, while Schechter et al. (1990) observed kidney uptake to be as high as 80% id/g. Schechter et al. (1990) proposed that the high kidney uptake of streptavidin may have to do with the presence of truncated forms of streptavidin in the preparations. Commercial preparations of streptavidin have been reported to contain truncated forms of streptavidin, generated as a result of certain isolation procedures performed during production. During these procedures, streptavidin apparently can undergo proteolytic degradation, resulting in the cleavage of 12-14 amino acid residues at the N-terminus and a maximum of 18 residues at the C-terminus (BAYER et al., 1986). Although not completely understood, it is hypothesized that the exposed amino acid residues resulting from truncation may interact with tissues like the kidneys, consequently resulting in high kidney
uptake of streptavidin (Schechter et al. 1990). A commercial preparation of streptavidin was used in this study, as well as in the studies performed by Hnatowich et al. (1993) and Schechter, et al. (1990). However, the streptavidin preparations came from different manufacturers in all three studies. Interestingly, when streptavidin was conjugated to the CC49 mAb, the kidney uptake of radioactivity was 35-fold lower. This suggests that by being conjugated to the mAb, streptavidin was somehow prevented from interacting with portions of the kidneys that, otherwise, would mediate uptake of the streptavidin protein.

The T:NT ratios for DOTA-biotin, at both time intervals, were ≥1:1, with exception of the tumour: kidneys ratios (Table 8), suggesting that the 2-step pre-targeting approach achieved tumour localization of DOTA-biotin. The tumour: kidneys ratios for both time intervals were ≤0.5:1 and are a result of the relatively high uptake of radioactivity by the kidneys. For both time intervals, the kidneys represented the highest uptake of radioactivity at 1.26-2.10% id/g, possibly reflecting the renal excretion of DOTA-biotin. The T:NT ratios for the DOTA-biotin control suggest that it achieved some non-specific tumour localization (Table 8). For example, the T:BL ratio for the DOTA-biotin control was 14:1, which is 5-fold higher than that of DOTA-biotin following pre-targeting with the 40 hour time interval. However, this is largely because blood levels of radioactivity following administration of the DOTA-biotin control were extremely low (0.01 ± 0.003% id/g) (Table 7). Tumour uptake (% id/g) of the DOTA-biotin control was actually 5-fold less than that of DOTA-biotin following pre-targeting with the 40 hour time interval.

After the 72 hour time interval, levels of $^{125}$I radioactivity were significantly lower in the blood and liver, suggesting that the longer time interval allowed for greater elimination of streptavidin-CC49 from these tissues to occur (Table 5). For the remaining normal tissues and tumour, however, lower levels of streptavidin-CC49 after the 72 hour time interval emerged only as a trend. This trend suggests that the 72 hour time interval may have allowed for greater elimination of antibody from these normal tissues to occur but, it may have also resulted in less tumour-bound streptavidin-CC49 available for binding to DOTA-biotin. No time interval
shorter than 40 hours was investigated, and therefore, the extent of antibody elimination from
the blood and normal tissues achieved over the first 40 hours pi is unknown. Nevertheless, to
deliver as much $^{90}$Y-DOTA-biotin and as high a radiation dose to the tumour as possible, 40
hours appears to be the appropriate time interval for the pre-targeted RIT study.

Figure 10A demonstrates that administering higher doses of DOTA-biotin increased
DOTA-biotin uptake in the tumour, as is evident by the increasing DOTA-biotin:streptavidin-
CC49 ratios per g of tumour. By using dual label techniques, we were able to measure the
DOTA-biotin:streptavidin-CC49 ratios per g of tumour, which in turn, allowed us to determine
which dose of DOTA-biotin achieved saturation of the biotin binding sites in the tumour.
Without normalization for blood radioactivity, the DOTA-biotin:streptavidin-CC49 ratios per g
of tumour increased almost linearly with the DOTA-biotin dose (Figure 10A). For the 1 μg
dose, the mean ratio was $0.19 \pm 0.06$ and for the 150 μg dose, $9.28 \pm 3.39$. In theory, these
ratios should reach a plateau with increasing dose because of the finite amount of biotin binding
sites available in the tumour due to using a fixed dose of streptavidin-CC49 (250 μg).
Theoretically, if conjugation of one streptavidin molecule per CC49 mAb was achieved, the
maximum DOTA-biotin:streptavidin ratios per g of tumour achievable would be 4:1 due to the
tetravalent binding between biotin and streptavidin. On the other hand, if conjugation of two
molecules of streptavidin per CC49 mAb was achieved, the maximum ratios achievable would
be 8:1. Either scenario is possible since the molecular weight of streptavidin-CC49 was
estimated by SDS-PAGE (Lane C, Figure 8) and size-exclusion HPLC (Figure 9) to be
between 200-300 kDa, i.e. IgG mAbs have a molecular weight of 150 kDa, while streptavidin
has weight of 65 kDa.

Increasing the DOTA-biotin dose would also increase the amount of DOTA-biotin
present in the blood at the time of analysis, either as free DOTA-biotin or DOTA-biotin
complexed with circulating streptavidin-CC49. To correct for the contribution of radioactive
DOTA-biotin in the blood circulating through the tumour, the absolute tumour uptake of
DOTA-biotin (pmols/g of tumour) was normalized to the absolute amount of DOTA-biotin in
blood (pmols/g of blood) (see: Appendix VI for calculations). Similarly, the tumour uptake of streptavidin-CC49 was normalized to the amount of streptavidin-CC49 in the blood. When corrected for blood radioactivity, the DOTA-biotin:streptavidin-CC49 ratios per g of tumour no longer increased linearly with the DOTA-biotin dose but rather, reached a plateau as the dose was increased from 40 to 150 µg (Figure 10B). The maximum ratio achieved at the 150 µg dose was 1.94:1. Pharmacokinetic modeling by van Osdol et al. (1993), suggested that equilibration of the biotin binding sites in a tumour nodule, pre-targeted with streptavidinylated mAb, could be reached within 4 hours of administration of biotin. The plateau observed with increasing doses of DOTA-biotin suggests that saturation of the biotin binding sites in the LS174T xenografts was achieved, both within the 6 hours elapsed since administration of DOTA-biotin and within the range of the DOTA-biotin doses administered. The DOTA-biotin:streptavidin-CC49 ratios per g of tumour (with normalization for blood radioactivity), obtained from increasing doses of DOTA-biotin, were significantly different from each other (p<0.0001). It appears that the most effective dose for the pre-targeted RIT study is 40 µg. This dose approached near saturation of the biotin binding sites, and in addition, will allow for DOTA-biotin to be administered at a higher specific activity, ie. µCi/µg, than will a dose of 150 µg. Higher specific activity is desirable since only a finite amount of DOTA-biotin can bind to the tumour-bound streptavidin-CC49. Tumour uptake of DOTA-biotin radiolabeled at higher specific activities could increase the radiation dose delivered to the tumour.

Our results have shown that tumour localization of DOTA-biotin with pre-targeting by streptavidin-CC49 was achieved. Moreover, we developed a dosing schedule for the pre-targeted RIT study, which involves administration of 250 µg of streptavidin-CC49, followed by a 40 hour time interval, and 40 µg of ⁹⁰Y-DOTA-biotin. The time interval of 40 hours and DOTA-biotin dose of 40 µg were chosen because they met specific criteria. Compared to the 72 hour time interval, the 40 hour interval demonstrated a trend towards higher tumour levels of streptavidin-CC49. The 40 µg dose of DOTA-biotin appears to have been sufficient for
saturating the biotin binding sites in the tumour. However, whether or not these parameters will result in RIT success can only be answered after the pre-targeted RIT study is carried out.
EXPERIMENT III:

PRE-TARGETED RADIOIMMUNOTHERAPY WITH STREPTAVIDIN-CC49 MONOClonAL ANTIBODY AND $^{89}$Y-DOTA-BIOTIN
Introduction

Pre-targeted RIT is an approach designed to be less toxic to bone marrow than conventional RIT with radiolabeled antibodies. Pre-clinical (Axworthy et al., 1995) and clinical (Cremonesi et al., 1997; Paganelli et al., 1993) investigations of pre-targeted RIT with the (strept)avidin-biotin system have yielded promising results. For example, Axworthy et al. (1995) administered up to 800 μCi of 90Y-DOTA-biotin to tumour-bearing mice following pre-targeting with streptavidylated mAb. Complete tumour regressions were seen in 8/10 treated mice bearing human breast tumours and in 10/10 treated mice bearing human colorectal and small cell lung carcinoma tumours. Dosimetry estimates predicted that tumour doses of >150 Gy/800 μCi were achieved. No apparent toxicity was observed with pre-targeting, however, conventional treatment with 400 μCi of 90Y-mAb was associated with 100% lethality in all mice and was less therapeutically effective than pre-targeted RIT. In a clinical study by Paganelli et al. (1993), five patients were treated with 40-50 mCi of 90Y-DOTA-biotin in a 3-step pre-targeting approach and demonstrated negligible hematological toxicity. Subsequently, 4/5 patients received a second course of treatment under a similar protocol with 100 mCi of 90Y-DOTA-biotin. Estimated bone marrow doses were 0.07 and 0.23 Gy for the first and second treatments, respectively. One patient demonstrated a minor response, one had stable disease, and three had progressive disease.

The objective of this experiment was to evaluate the therapeutic efficacy and safety of pre-targeted RIT with streptavidin-CC49 and 90Y-DOTA-biotin, using the dosing schedule previously developed in Experiment II. The dosing schedule involves ip administration of streptavidin-CC49 at a dose of 250 μg, followed by iv administration of 40 μg of 90Y-DOTA-biotin 40 hours later. The 40 hour time interval was chosen because it demonstrated a trend towards greater tumour retention of streptavidin-CC49 compared to the 72 hour time interval. The 40 μg dose of DOTA-biotin appeared to be the optimal dose for saturating the hapten binding sites in the tumour and for delivering as high a radiation dose as possible to the tumour compared to other DOTA-biotin doses of 1, 20, and 150 μg. Radiolabeled CC49 mAb has
previously been shown in animal models to be effective in treating tumours. A 5-fold reduction in tumour volume was observed following the administration of 300 μCi of $^{131}$I-CC49 mAb to mice bearing HT-29 xenografts (Greiner et al., 1993). In a subsequent study, Greiner et al. (1994) treated HT-29 tumour-bearing mice with multiple doses (2x) of 300 μCi of $^{131}$I-CC49 mAb and dramatically inhibited tumour growth for the first 21 days after treatment. Not surprisingly, clinical RIT with CC49 mAb has shown less promising results than animal studies, as has clinical RIT of most solid tumours. In a Phase I trial, twenty-four patients were treated with $^{131}$I-CC49 mAb at doses of 15-90 mCi/m² (Divgi et al., 1995). Only stable disease and one minor response were observed, with hematological toxicity appearing at doses >60 mCi/m². Using the dosing schedule developed in Experiment II, athymic mice bearing subcutaneous human LS174T xenografts were treated with 900 μCi of $^{90}$Y-DOTA-biotin as part of a small pilot study. Tumour growth, peripheral white blood cell (WBC) counts, and whole body weights were evaluated over a period of 25 days following the administration of $^{90}$Y-DOTA-biotin to assess the therapeutic efficacy and toxicity of the pre-targeted RIT regimen.

**Methods**

**Preparation of Streptavidin-CC49 Monoclonal Antibody**

CC49 mAb was purified from mouse ascites fluid and analyzed for purity by size-exclusion HPLC and SDS-PAGE (see: Appendix I). CC49 mAb was then conjugated to streptavidin and the conjugate evaluated for purity and molecular weight by size-exclusion HPLC and SDS-PAGE (see: Appendix II). The immunoreactive fraction of streptavidin-CC49 was 63% (see: Appendix III). Prior to administration, streptavidin-CC49 was sterilized by passing the antibody solution through a 0.22 μm HT Tuffryn membrane syringe filter (Gelman Sciences, Ann Arbor, MI) and then tested for sterility by the USP XXIII sterility test. Tests results were negative for bacterial and fungus contamination.
Preparation of $^{90}$Y-DOTA-biotin (see: Appendix IV)

DOTA-biotin was labeled with $^{90}$Y chloride to a specific activity of approximately 22-23 $\mu$Ci/µg. The radiochemical purity of $^{90}$Y-DOTA-biotin assessed by reversed-phase chromatography was 99% and the streptavidin-binding capacity was 92%. To minimize radiolysis of DOTA-biotin, ascorbic acid (20 mg/mL) (Fisher Scientific, Markham, Ont.) was added. DTPA calcium trisodium salt hydrate (10 mM) (Fluka Chemika, Switzerland) was also added to chelate any free $^{90}$Y ions present in the preparation. Yttrium 90-DOTA-biotin was diluted for injection with sterile 0.9% saline and the final solution was sterilized as previously described. Yttrium 90-DOTA-biotin was also tested for sterility by the USP XXIII sterility test and was negative for bacterial and fungal contamination.

Radioimmunotherapy with Streptavidin-CC49 Monoclonal Antibody and $^{90}$Y-DOTA-biotin

LS174T human colon cancer cells were injected subcutaneously in twelve six- to eight-week old female athymic mice and the tumours allowed to grow for 12 days, at which time tumour diameters ranged from 3-6 mm (see: Appendix V). One group of tumour-bearing mice (n=6) received 250 µg of streptavidin-CC49 by ip injection and 40 hours later received 900 $\mu$Ci (40 µg) of $^{90}$Y-DOTA-biotin by tail vein injection. Prior to performing tail vein injections of $^{90}$Y-DOTA-biotin, all mice were anesthetized as previously described in Experiment I. The remaining tumour-bearing mice served as the control group (n=6) and received no treatment. Tumour volumes in both groups of mice were measured 2-3 times a week for 25 days following treatment with $^{90}$Y-DOTA-biotin, and were calculated using the following formula: volume (mm$^3$) = $4/3\pi[(r_1+r_2) - 2]^3$, where $r_1$ and $r_2$ are the radii of the long and short axes of the tumour measured by precision calipers. Bone marrow toxicity was evaluated by measuring peripheral WBC in all mice 2 or 3 times a week for 25 days following treatment. Peripheral WBC counts were performed by withdrawing whole blood via the tail vein using 47.7 µL-sized heparinized microcapillary tubes. Prior to the collection of blood samples, all mice were anesthetized as previously described in Experiment I. Blood samples were diluted 1:20: v/v
with Turk's solution containing 3% acetic acid and 0.01% Crystal Violet stain (Sigma Chemical Co.) and incubated for at least 25 minutes at room temperature. White blood cells were then counted on a standard hemocytometer. Toxicity was also monitored by measuring the whole body weight of each mouse 2 or 3 times a week for 25 days following treatment. Throughout the entire study, the mice were housed in sterile cages fitted with filtered lids and maintained on a diet of sterile water and rodent chow (Autoclavable Rodent Chow 5010, PMI Feeds, St. Louis, MO).

Statistical Analysis

The mean tumour volume was compared between groups using the Student's unpaired t-test with a level of significance of p<0.05.

Results

Size-exclusion HPLC of CC49 mAb purified from mouse ascites fluid showed a single peak with retention times of 8.3-8.6 minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified CC49 mAb showed only one protein band identified as having a molecular weight between 116-200 kDa. Size-exclusion HPLC of streptavidin-CC49 revealed two peaks with retention times of 7.3-7.6 minutes and 8.2-8.3 minutes, representing streptavidin-CC49 and unconjugated CC49 mAb, respectively. Two bands representing streptavidin-CC49 and unconjugated CC49 mAb corresponding to molecular weights >200 kDa and between 116-200 kDa, respectively, were identified by SDS-PAGE. Both size-exclusion HPLC and SDS-PAGE did not detect the presence of any unconjugated streptavidin. Analysis of the size-exclusion HPLC chromatograms of streptavidin-CC49, as previously described in Experiment II, revealed that approximately 50% of CC49 mAb present in the streptavidin-CC49 preparation remained unconjugated.

At the time of streptavidin-CC49 administration tumour volumes ranged from 8-268 mm$^3$ (mean=81 mm$^3$) and from 48-113 mm$^3$ (mean=74 mm$^3$) for mice in the untreated and
treated groups, respectively. One mouse from the untreated group was sacrificed on the seventh day following treatment with $^{90}$Y-DOTA-biotin, due to significant ulceration of the tumour. On days 11 and 14 one mouse from the treated group and one mouse from the untreated group, respectively, died from anesthetic overdose. Another death, also attributed to anesthetic overdose, occurred in the treated group on day 22.

Figure 11A shows individual tumour growth curves for all mice measured over the 25 day period following treatment with $^{90}$Y-DOTA-biotin, expressed as the % change in tumour volume. The % change in tumour volume was calculated as: $(\text{tumour volume}_{\text{day x}} - \text{tumour volume}_{\text{day 0}}) / \text{tumour volume}_{\text{day 0}} \times 100\%$. One mouse (#3) from the treated group demonstrated a noticeable response to therapy. After an initial increase in tumour volume of approximately 40% during the first 3 days following treatment, no further measurable tumour growth was observed until 11 days later on day 14. On day 16 the tumour appeared to regress, reaching a volume on day 20 that was approximately 50% smaller than its volume prior to $^{90}$Y-DOTA-biotin administration, i.e. on day 0. However, on day 22 tumour growth resumed, and at the end of the study on day 25, the tumour volume was $110\%$ larger than that measured on day 0. Figure 12 shows the anterior views of an untreated mouse (#6) and the treated mouse described above (#3) taken on day 25. On day 0 the tumour volumes were 180 mm$^3$ and 144 mm$^3$ for mouse #3 and mouse #6, respectively. However, on day 25 the tumour volume of mouse #6 was approximately 1680 mm$^3$, whereas the tumour volume of mouse #3 treated with streptavidin-CC49 and 900 μCi of $^{90}$Y-DOTA-biotin was nearly 6-fold smaller at 268 mm$^3$. The tumour volume of mouse #6 increased by $1000\%$ from day 0, nearly 10-fold greater than the change in tumour volume observed with mouse #3. Tumour growth rates of the remaining treated mice were similar to most of those of the untreated mice (Figure 11A). Figure 11B shows the mean % change in tumour volume over time for both groups of mice, excluding all the mice that died or were sacrificed prior to the completion of the study. At the end of the study on day 25, the mean % change in tumour volume was $1974 \pm 1475\%$ (n=4) and $692 \pm$
Figure 11. Tumour growth curves following pre-targeted radioimmunootherapy. LS174T tumour-bearing mice were: i) treated with 250 μg of streptavidin-CC49 by intraperitoneal injection and 40 hours later were administered 900 μCi (40 μg) of $^{90}$Y-DOTA-biotin by tail vein injection (open circles) or ii) left untreated (closed circles). (A) Individual tumour growth curves including all mice that died or were sacrificed prior to the completion of the study; (B) Mean tumour growth curves ± SD (n = 4 mice/group) excluding all mice that died or were sacrificed prior to the completion of the study. Tumour growth curves are expressed as the % change in tumour volume, calculated as: $\frac{(\text{tumour volume}_{\text{day}x} - \text{tumour volume}_{\text{day}y})}{\text{tumour volume}_{\text{day}y}} \times 100\%$.

a Arrow denotes administration of streptavidin-CC49.
b Arrow denotes administration of $^{90}$Y-DOTA-biotin.
Figure 12. Suppression of 1.S174T tumour growth following pre-targeted radioimmunotherapy. 1.S174T tumour-bearing mice were: i) treated with 250 µg of streptavidin-C649 by intraperitoneal injection and 40 hours later were administered 900 µCi (40 µg) of 90Y-DOTA-biotin by tail vein injection or ii) left untreated. Photographs were taken on day 25 at the completion of the study. Tumours are located in the left lower abdominal region of the mice. (A) Anterior view of mouse #6 (untreated). Tumour volume = 1680 mm³; (B) Anterior view of mouse #3 (treated). Tumour volume 268 mm³.
410% (n=4) for the untreated and treated mice, respectively. These values were not statistically different. Tumour volumes are also presented in Table 9.

The mean peripheral WBC counts for either group was highly variable throughout the 25 day evaluation period but, there appeared to be no overall decline in WBC counts in the treated mice that would indicate WBC depression (Figure 13A). Whole body weights for both groups of mice varied to a lesser extent than the mean WBC counts (Figure 13B). The mean body weights of treated and untreated mice also did not decline during the 25 day period, indicating no apparent deterioration of health in the animals.

Discussion

Pre-targeted RIT is an approach which, compared to conventional RIT with radiolabeled mAbs, may reduce bone marrow toxicity and possibly allow higher amounts of radioactivity to be safely administered to the patient. Several pre-clinical and clinical investigations of pre-targeted RIT with the (strept)avidin-biotin system have been performed and have yielded encouraging results (Axworthy et al., 1995; Cremonesi et al., 1997; Paganelli et al., 1993). In this small pilot study, we evaluated the therapeutic efficacy and toxicity of streptavidin-CC49 and 90Y-DOTA-biotin in athymic mice bearing subcutaneous human LS174T colorectal xenografts. We administered 250 µg of streptavidin-CC49 by ip injection and 40 hours later, administered 900 µCi (40 µg) of 90Y-DOTA-biotin by iv injection. Following treatment with 90Y-DOTA-biotin, tumour growth, peripheral WBC counts, and whole body weights were evaluated over a 25 day period following the administration of 90Y-DOTA-biotin to evaluate the therapeutic efficacy and toxicity of the pre-targeted RIT approach.

It has been postulated that a radiation dose of at least 50-80 Gy must be delivered to the tumour within a period of 1 week for successful treatment (Bruland, 1995; Ellis, 1968). Only 1/6 treated mice demonstrated a major response to therapy (Figure 11A; Figure 12), possibly indicating that cytotoxic doses of radiation may not have been delivered to all the tumours treated with the streptavidin-CC49 and 90Y-DOTA-biotin regimen. Nevertheless, at
Table 9. Individual tumour volumes of LS174T xenografts following treatment with streptavidin-CC49 and $^{90}$Y-DOTA-biotin.

<table>
<thead>
<tr>
<th>Day Post-treatment</th>
<th>Tumour Volume (mm$^3$)</th>
<th>Treated Mice</th>
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<tbody>
<tr>
<td></td>
<td>Untreated Mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1$^0$</td>
<td>2$^Y$</td>
</tr>
<tr>
<td>-1.6$^+$</td>
<td>87</td>
<td>268</td>
</tr>
<tr>
<td>0$^+$</td>
<td>180</td>
<td>449</td>
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<tr>
<td>25</td>
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LS174T tumour-bearing mice were: i) treated with 250 μg of streptavidin-CC49 by intraperitoneal injection and 40 hours later were administered 900 μCi (40 μg) of $^{90}$Y-DOTA-biotin by tail vein injection or ii) left untreated.

$^+$ Streptavidin-CC49 administered.

$^\dagger$ $^{90}$Y-DOTA-biotin administered.

$^\circ$ Mouse sacrificed on day 7 due to significant ulceration of the tumour.

$^\circ$ Mice died from anesthetic overdose on days indicated by "*".
Figure 13. Evaluation of $^{90}$Y-DOTA-biotin toxicity following pre-targeted radioimmunotherapy. LS174T tumour-bearing mice were: i) treated with 250 μg of streptavidin-CC49 by intraperitoneal injection and 40 hours later were administered 900 μCi (40 μg) of $^{90}$Y-DOTA-biotin by tail vein injection (open circles) or ii) left untreated (closed circles). (A) Mice were evaluated for myelosuppression by monitoring peripheral white blood cell counts; (B) Whole body toxicity was evaluated by monitoring whole body weights. Data points represent mean values ± SD (n=4 mice/group). All mice that died or were sacrificed prior to the completion of the study were excluded.

a Arrow denotes administration of streptavidin-CC49.
b Arrow denotes administration of $^{90}$Y-DOTA-biotin.
the completion of the 25 day evaluation period, the mean % change in tumour volume for the untreated mice was 1974 ± 1475% (n=4), whereas that of the treated mice was 692 ± 410% (n=4) (Figure 11B). These values were not significantly different, however, the trend suggests that the treatment regimen may have had a therapeutic effect. Perhaps with larger sample sizes, a significant difference may be observed. The lack of apparent toxicity at the dose of 90Y-DOTA-biotin administered (900 µCi) may allow for larger doses of 90Y-DOTA-biotin to be safely administered in order to achieve a higher response rate. Alternatively, a second course of treatment with streptavidin-CC49 and 90Y-DOTA-biotin 1-2 weeks following the initial course of therapy could be given. One concern with this approach, however, is the possibility of HAMA and HASA responses in patients, which may diminish the effectiveness of the treatment.

The mean peripheral WBC counts for both groups of mice were highly variable, possibly due to experimental error (Figure 13A). There was no obvious decline in the mean peripheral WBC counts in the treated mice compared to pre-treatment values but, perhaps with larger sample sizes the variability would be less and the changes in WBC counts more easily discernible. Based on the mean peripheral WBC counts, there was no apparent bone marrow toxicity following the administration of 900 µCi of 90Y-DOTA-biotin. In mice, myelosuppression has typically been observed within 14 days from the time of treatment with radiolabeled mAb (Lee et al., 1990; Senekowitsch et al., 1989; Buchegger et al., 1990). Therefore, the length of the evaluation period (25 days) should have been sufficient to observe myelosuppression. Mean body weight measurements were not as variable as the mean peripheral WBC counts and, it was quite apparent that no loss in body weight occurred in the treated mice, suggesting that the health of the animals did not deteriorate (Figure 13B). If myelosuppression did occur but, was undetected, the fact that the mice were still able to thrive following the administration of 90Y-DOTA-biotin suggests that bone marrow toxicity, if any, was not life-threatening. No comparison was made between the therapeutic efficacy and toxicity of pre-targeted RIT to that of conventional RIT with radiolabeled mAbs in this study.
However, comparisons with other studies support the hypothesis that pre-targeted RIT may be less toxic to bone marrow than conventional RIT. Lee et al. (1990), observed a 10-fold decrease in WBC counts in mice within 2 weeks of administering only 150-250 μCi of 90Y-CO17-1A mAb. Sharkey et al. (1988) observed as much as a 50-85% decrease in WBC counts in mice within 1-3 weeks following therapy with 20 and 50 μCi of 90Y-NP2 mAb. Results from a pre-clinical study by Axworthy et al. (1995) further support our findings and the hypothesis that pre-targeted RIT may be less toxic to bone marrow. These authors observed no apparent bone marrow toxicity in tumour-bearing mice treated with 200-800 μCi of 90Y-DOTA-biotin after pre-targeting with streptavidinylated mAb. Conventional RIT with 400 μCi of 90Y-mAb, however, resulted in 100% lethality to the animals. Moreover, the conventional RIT approach was not as therapeutically effective as the pre-targeted approach, which achieved complete tumour regressions in as high as 10/10 mice bearing colorectal and small cell lung carcinoma xenografts. In this study, pre-targeted RIT of LS174T colorectal xenografts was also not as therapeutically effective as the pre-targeted RIT performed by Axworthy et al. (1995). The dosing regimen employed in this study may not necessarily be the optimal regimen for pre-targeted RIT in this animal model, and therefore, it may be essential to investigate and compare the therapeutic efficacy and toxicity of other dosing regimens, i.e., those with a shorter time interval or different doses of antibody and effector molecule.

Results from this small pilot study are encouraging because of the considerable suppression in tumour growth achieved in at least one animal. Despite the relatively high dose of 90Y-DOTA-biotin administered (900 μCi), the treated mice continued to thrive and no apparent bone marrow toxicity was observed. Furthermore, while only 1/6 mice treated with streptavidin-CC49 and 90Y-DOTA-biotin demonstrated a considerable response to therapy, the observed trend towards a smaller % change in tumour volume following treatment suggests that the treatment regimen may have had a therapeutic effect. However, larger samples sizes will be required in future evaluations to confirm the therapeutic efficacy and bone marrow toxicity of this or any similar pre-targeted RIT dosing regimen. Possibilities for increasing the
Response rate include administering higher doses of $^{90}$Y-DOTA-biotin (>900 μCi), performing multiple courses of treatment with streptavidin-CC49 and $^{90}$Y-DOTA-biotin, and using other dosing regimens. Nevertheless, pre-targeted RIT with streptavidin-CC49 and $^{90}$Y-DOTA-biotin has demonstrated some promise as an effective therapeutic approach to the treatment of colon cancer but, additional pre-clinical studies are necessary (see: Conclusions and Future Perspectives), before its clinical efficacy and safety can be evaluated in patients.
CONCLUSIONS AND FUTURE PERSPECTIVES

Two major limitations of RIT are the sub-therapeutic radiation doses delivered to solid tumours and the bone marrow toxicity in the form of myelosuppression. Reducing bone marrow toxicity is essential in improving the clinical success of RIT because it may also permit higher amounts of radioactivity to be safely administered to the patient, which in turn, may improve the radiation doses delivered to the tumour. We investigated a novel RIT approach designed to reduce bone marrow toxicity, which involves pre-targeting of the lesion with a streptavidinylated mAb followed by administration of radiolabeled biotin. The overall objective of our study was to investigate pre-targeted RIT with streptavidin-CC49 mAb and $^{90}$Y-DOTA-biotin in athymic mice bearing subcutaneous human LS174T xenografts.

In Experiment I, we demonstrated that DOTA-biotin is suitable for pre-targeting strategies. Blood levels of DOTA-biotin, labeled with $^{111}$In, in BALB/c mice were <2% id/g at 1 hour pi (Figure 5). Blood levels of radioactivity decreased rapidly by 5- and 100-fold at 2 and 12 hours pi, respectively. Kidneys demonstrated the highest uptake of radioactivity at 3.2% id/g at 1 hour pi, which decreased by 8-fold to 0.4% id/g at 12 hours pi (Figure 6). This pattern of uptake and elimination of radioactivity in the kidneys suggested that DOTA-biotin was eliminated by renal excretion. In the remaining normal tissues, uptake of radioactivity was very low and was measured at <0.3% id/g at 3 hours pi (Figure 6).

In Experiment II, a dosing schedule for pre-targeted RIT with streptavidin-CC49 and $^{90}$Y-DOTA-biotin was developed. Dual label biodistribution studies in LS174T tumour-bearing mice allowed us to determine the time interval and DOTA-biotin dose to be used in the RIT study. The 40 hour time interval demonstrated a trend towards greater tumour retention of streptavidin-CC49 compared to the 72 hour time interval ($6.81 \pm 3.08$% id/g vs. $4.26 \pm 1.22$% id/g), and hence, was chosen as the time interval for the pre-targeted RIT study (Table 5). When normalized for blood radioactivity, the DOTA-biotin dose of 40 $\mu$g appeared to have reached near saturation of the biotin binding sites in the tumour, achieving a DOTA-
biotin:streptavidin-CC49 ratio per g of tumour of 1.33:1 (Figure 10B). Therefore, the 40 μg dose was chosen as the dose for the pre-targeted RIT study.

A small pilot study was carried out in Experiment III evaluating the therapeutic efficacy and toxicity of pre-targeted RIT with streptavidin-CC49 and ⁹⁰Y-DOTA-biotin. Using the dosing schedule developed in Experiment II, athymic mice bearing subcutaneous human LS174T xenografts were treated with 900 μCi of ⁹⁰Y-DOTA-biotin. Only 1/6 treated mice demonstrated a considerable suppression in tumour growth (Figure 11A; Figure 12). In this mouse, no further measurable tumour growth was observed over a period of 11 days. The tumour did appear to regress but, shortly thereafter, tumour growth resumed. The tumour growth rates of the remaining treated mice were similar to those of most of the untreated mice, however, the trend towards a smaller % change in tumour volume following treatment suggested that the treatment regimen may have had a therapeutic effect. At the completion of the study, the mean % change in tumour volume for the treated mice was 610 ± 410% (n=4) and that of the untreated mice was 1974 ± 1475% (n=4) (Figure 11B). At the relatively high dose of ⁹⁰Y-DOTA-biotin administered (900 μCi), no apparent bone marrow toxicity or deterioration in health of the animals were observed (Figure 13).

Our results demonstrated that streptavidin-CC49 and ⁹⁰Y-DOTA-biotin have some promise as an effective RIT approach to treat colorectal cancer. Despite only observing one considerable response to therapy, the lack of apparent bone marrow toxicity and deterioration in health suggested that pre-targeted RIT with the (strept)avidin-biotin system may be less toxic to bone marrow than conventional RIT approaches. The pre-targeting approach, therefore, may also permit higher amounts of radioactivity to be administered to a patient, and perhaps, improve the radiation dose delivered to the tumour. Hence, pre-targeting has the potential to achieve greater clinical success than conventional RIT approaches.

Utilizing other mAbs can further extend our dosing regimen beyond treating colorectal cancer. Alternatively, CC49 mAb, a pan-carcinoma antibody, can potentially be used to treat other adenocarcinomas such as, ovarian and breast cancer. The pre-targeting approach is more
complicated than conventional RIT with radiolabeled mAbs because of time intervals to consider and the additional reagents for which optimal doses must be determined. The dosing regimen employed in this study may not have necessarily been the optimal regimen for treating LS174T xenografts. Future perspectives, therefore, for pre-targeted RIT with the (strept)avidin-biotin system may include further pre-clinical evaluation of this approach using different dosing regimens, ie. shorter time intervals, different doses of antibody, or multiple treatment protocols.
REFERENCES


LIST OF PUBLICATIONS AND ABSTRACTS


APPENDIX I

Purification of CC49 Monoclonal Antibody From Mouse Ascites Fluid

Mouse ascites fluid was generously provided by Dr. J. Schlom of The National Cancer Institute, Bethesda, Maryland. CC49 mAb was purified from ascites fluid by affinity chromatography using a Protein G agarose column (Pierce, Rockford, IL). Following purification from ascites fluid, CC49 mAb was desalted on a PD-10 Sephadex G-25 column (Pharmacia, Baie d'Urfe, Québec) eluted with sodium bicarbonate buffer (50 mM, pH 7.5) and then concentrated to 10 mg/mL in a Centricon-30 concentrator (Amicon, Beverly, MA). The purity of CC49 mAb was evaluated by size-exclusion HPLC on a Bio-Silect 250-5 column (Bio-Rad, Richmond, CA) eluted with phosphate buffer (50 mM, pH 6.5) at 1 mL/minute. Protein was identified by using an in-line UV absorbance monitor (Model 2141, LKB, Bromma, Sweden) set at 215 nm. The molecular weight of the purified protein was estimated using a standard curve, generated from the retention times of protein standards. The protein standards (Bio-Rad) included: tyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.3 kDa), which had retention times of approximately 6.4, 8.8, 9.5, 11.4, and 13.6 minutes, respectively. CC49 mAb purity was also evaluated by SDS-PAGE using a 4-20% Tris-glycine gel (Bio-Rad). Protein bands were identified by Coomassie R-250 stain.
APPENDIX II

Preparation of Streptavidin-CC49 Monoclonal Antibody

CC49 mAb purified from mouse ascites fluid was conjugated to streptavidin (Sigma Chemical Co., St. Louis, MO) using sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce Chemical Co.), a protein cross-linker molecule (Ngai et al., 1995; Alvarez-Diez et al., 1996). Briefly, disulphide bridges on the CC49 mAb (10 mg/mL) were reduced by incubation with mercaptoethanolamine (Sigma Chemical Co.) at a mercaptoethanolamine:CC49 mAb molar ratio of 6,000:1 in phosphate buffered saline (PBS) (50 mM; pH 7.3) for 1 hour and 30 minutes at a temperature of 37°C. Streptavidin (1 mg/mL) was reacted with sulfo-SMCC at a sulfo-SMCC:streptavidin molar ratio of 1,440:1 in PBS for 30 minutes at a temperature of 37°C. Reduced CC49 mAb and maleimide-derivatized streptavidin were then purified from excess reagents on a Sephadex G-50 mini-column eluted with PBS. Sephadex G-50 mini-columns were prepared by packing glass Pasteur pipettes, plugged at the neck with glass wool, with Sephadex G-50 resin (Sigma Chemical Co.). After purification, reduced CC49 mAb and maleimide-derivatized streptavidin were reacted together overnight at room temperature. The molecular weight and purity of the conjugate were evaluated by size-exclusion HPLC and by SDS-PAGE, as previously described in Appendix I.
APPENDIX III

Measurement of the Immunoreactive Fraction of Streptavidin-CC49 Monoclonal Antibody

The immunoreactive fraction of streptavidin-CC49 was determined by measuring the fraction of radiolabeled conjugate bound to CNBr-activated Sepharose 4B beads (Sigma Chemical Co.) that were previously covalently linked to bovine submaxillary mucin (BSM) (Sigma Chemical Co.), a known source of the TAG-77 antigen. Briefly, streptavidin-CC49 was incubated with $^{111}$In-DOTA-biotin for 30 minutes at a temperature of 37°C and the unbound $^{111}$In-DOTA-biotin was removed by filtration using Centricon-30 concentrators. Approximately 30 ng of streptavidin-CC49, labeled with $^{111}$In-DOTA-biotin, was incubated with 125 μL of BSM-coated Sepharose beads in a 0.45 μm MicroPure Filter (Amicon) for 45 minutes at room temperature under gentle agitation. At the end of the incubation period, the filter was counted for $^{111}$In radioactivity in a gamma well counter (Packard Model Auto-Gamma 5650, Downer's Grove, IL) using a window of 150-260 keV to include the 172 keV and 240 keV γ-photons of $^{111}$In. The filter was then centrifuged and the filtrate containing unbound streptavidin-CC49 labeled with $^{111}$In-DOTA-biotin was incubated with a new 125 μL aliquot of BSM-coated Sepharose beads, as previously described. After the completion of the second incubation, the filter was centrifuged, the filtrate discarded, and both filters containing BSM-coated Sepharose were counted for $^{111}$In radioactivity. Based on the known amount of radioactivity added to the BSM-coated Sepharose beads in the first incubation, the immunoreactive fraction of streptavidin-CC49 was measured as the total percentage of activity remaining on the filters.
APPENDIX IV

Preparation of $^{111}$In- and $^{90}$Y-DOTA-biotin

DOTA-biotin was generously provided by the NeoRx Corporation of Seattle, Washington. $^{111}$In- and $^{90}$Y-DOTA-biotin were prepared by incubating DOTA-biotin with $^{111}$In chloride (Nordion, Kanata, Ont.) or $^{90}$Y chloride (New England Nuclear, Boston, MA) in ammonium acetate buffer (500 mM, pH 5) for 60 minutes in a water bath heated to a temperature of 80°C. The radiochemical purity of $^{111}$In- and $^{90}$Y-DOTA-biotin was determined by reversed-phase chromatography using disposable C-18 Sep-Pak cartridges (Waters, Milford, MA). For pre-targeting studies, the streptavidin-binding capacity of radiolabeled DOTA-biotin was evaluated by incubating radiolabeled DOTA-biotin with a ≥4-fold molar excess of streptavidin-CC49 for 30 minutes at a temperature of 37°C. Streptavidin-CC49 bound to radiolabeled DOTA-biotin and free radiolabeled DOTA-biotin were separated by elution through a Sephadex G-50 mini-column with 0.9% saline. The eluate fractions were measured for $^{111}$In radioactivity in a gamma well counter (Packard Model Auto-Gamma 5650) as previously described in Appendix III. To measure $^{90}$Y radioactivity, the eluate fractions were measured for Bremstrahlung radiation in the gamma well counter using an open window of 50-1000 keV. The streptavidin-binding capacity was measured by comparing the total radioactivity eluted in the void volume fractions ($^{111}$In-$^{90}$Y-DOTA-biotin-streptavidin-CC49) to that eluted as free radiolabeled DOTA-biotin in the fractionation range eluates.
**APPENDIX V**

**Establishment of LS174T Colon Cancer Xenografts**

LS174T human colon cancer cells were obtained from the American Type Cell Culture (Rockville, MD) company and maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum (Sigma Chemical Co.) under 37°C/5% CO₂ conditions. For xenograft establishment, LS174T cells were recovered by trypsinization with 0.25% trypsin/EDTA (Sigma Chemical Co.) and resuspended to 3.0 x 10⁷ cells/mL in sterile 0.9% saline. Approximately 3.0 x 10⁶ LS174T cells in a 100 μL volume were injected subcutaneously in the lower left abdominal region of six- to eight-week old female Swiss nu/nu (athymic) mice (Charles River Laboratories, Boston, MA) and allowed to grow for 8-15 days prior to the start of the biodistribution and therapy studies. All mice were acclimatized for at least two weeks before xenograft establishment and were housed in sterile cages fitted with filtered lids. The mice were maintained on a diet of sterile water and rodent chow (Autoclavable Rodent Chow 5010, PMI Feeds, St. Louis, MO). All animal studies were conducted under an approved protocol from the Animal Care Committee at The Toronto Hospital following CCAC guidelines.
APPENDIX VI

Calculation of Absolute Streptavidin-CC49 and DOTA-biotin Uptake in LS174T Xenografts

**DOTA-biotin**

pmols DOTA-biotin/g of tumour \( (A) = \left( \frac{\% \ id/g \ of \ tumour}{100} \right) \times \text{mass of injectate} \) \( \times \ \frac{900 \ \text{Da}}{1 \times 10^{12} \ \text{pmols/mol}} \)

**Streptavidin-CC49 Monoclonal Antibody**

pmols streptavidin-CC49/g of tumour \( (C) = \left( \frac{\% \ id/g \ of \ tumour}{100} \right) \times \frac{0.5}{1 \times 10^{12} \ \text{pmols/mol}} \)

Therefore, DOTA-biotin:streptavidin-CC49 ratio \( = \frac{(A)}{(C)} \)

**DOTA-biotin (Normalized to Blood)**

pmols DOTA-biotin/g of blood \( (E) = \left( \frac{\% \ id/g \ of \ tumour}{100} \right) \times \frac{900 \ \text{Da}}{1 \times 10^{12} \ \text{pmols/mol}} \)

Therefore, absolute tumour uptake of DOTA-biotin (normalized to blood) \( (G) = \frac{(A)}{(E)} \)

**Streptavidin-CC49 Monoclonal Antibody (Normalized to Blood)**

pmols streptavidin-CC49/g of blood \( (H) = \left( \frac{\% \ id/g \ of \ tumour}{100} \right) \times \frac{215 \ 000 \ \text{Da}}{1 \times 10^{12} \ \text{pmols/mol}} \)

Therefore, absolute tumour uptake of streptavidin-CC49 (normalized to blood) \( (J) = \frac{(C)}{(H)} \)

Therefore, DOTA-biotin:streptavidin-CC49 ratio (normalized to blood) \( = \frac{(G)}{(J)} \)

\( \frac{\% \ id}{g} \) = 1 x 10^{-6}, 2 x 10^{-5}, 4 x 10^{-5}, or 1.5 x 10^{-4} g

\( \frac{\% \ id}{g} \) are 50% of injectate is unconjugated CC49 mAb

\( \frac{\% \ id}{g} \) mass = 2.5 x 10^{-4} g

Streptavidin-CC49 presumed to be made up of 1 streptavidin molecule (65 kDa) and 1 CC49 mAb (150 kDa). Therefore, total molecular weight = 215 kDa.