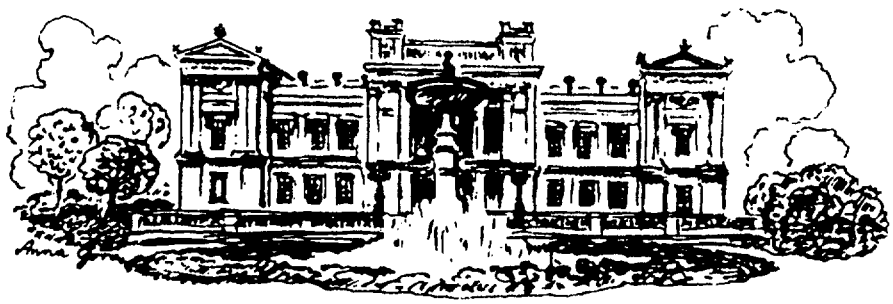
RADIOLABELING AND BIOTINYLATION OF INTERNALIZING MONOCLONAL ANTIBODY CHIMERIC BR96

Potential Use for Extracorporeal Immunoadsorption with Enhanced Tumor Radioactivity Retention of Iodine, Indium and Rhenium

陈建清

JianQing Chen



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JianQing Chen

LUND UNIVERSITY
THE JUBILEUM INSTITUTE
DEPARTMENT OF RADIATION PHYSICS,
S-221 85 LUND
SWEDEN

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Abstract

In this thesis, methodology of radiolabeling and simultaneous biotinylation for internalizing monoclonal antibody (MAb) chimeric BR96 (chiBR96) have been investigated by using three element groups of potential therapeutic radionuclides iodine, indium and rhenium, and their different labeling methods. The biodistribution and kinetics of biotinylated and radiolabeled chiBR96 have been studied in colon carcinoma isografted rats. The potential use of ECIA, based on the biotin-avidin concept, has been evaluated and compared with the approach of avidin "chase" in the same animal tumor model with respect to an enhancement of tumor-to-normal tissue (T/N) activity ratio.

In vivo stability and tumor targeting capacity of biotinylated ^{111}In-chiBR96 using the chelate SCN-Bz-CHX-A-DTPA, have increased compared with two other chelates used in this study. Conjugate NSTBB has been successfully used for iodination of chiBR96 combined with biotinylation, in contrast to the combination with electrophilic labeling methods which seem particularly difficult to use. The T/N activity ratio has been enhanced by using NSTBB conjugate, compared with the Iodogen method. ChiBR96 simultaneously biotinylated and ^{188}Re-labeled using chelate TFP MAG_2-GABA, demonstrates an activity distribution similar to radioiodine-chiBR96.

Through the direct removal of circulating radioimmunoconjugates, ECIA significantly enhances the T/N activity ratio for biotinylated ^{188}Re-chiBR96, and exhibits some advantages over "avidin" chase.

For internalizing MAb chiBR96, the tumor activity uptake and retention have been significantly increased by selecting the cellularly retained radionuclide ^{111}In, instead of iodine and rhenium. The radioactivity distributions of chiBR96, biotinylated and radiolabeled with ^{111}In, ^{125}I and ^{188}Re in normal tissue, are similar within 48 h post-injection. This demonstrates that ECIA has a considerable ability to remove the background of ^{111}In-labeled immunoconjugates with a prompt enhancement of T/N activity ratios.

Key words

Radialabeling, Biotinylation, Chimeric BR96, Extracorporeal Immunoadsorption, Avidin Chase.

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by

JianQing Chen

Akademisk avhandling som, för avläggande av filosofie doktorsexamen vid medicinska fakulteten vid Lunds Universitet, kommer att offentligen försvaras i onkologens föreläsningssal, Universitetssjukhuset i Lund, onsdagen den 5 februari 1997 kl 10.15.

Fakultetsopponent: Professor Alan R. Fritzberg, Seattle, USA

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1. LIST OF PAPERS

1. Chen J.Q., Strand S-E. and Sjögren H.O. Comparative *in vitro* and *in vivo* studies of the stability of ^{111}In-labeled, and biotinylated CDTA or DTPA monoclonal antibody conjugates. *Tumor Targeting 1995;1:331-9.*

2. Chen J.Q., Strand S-E., Brechbiel M.W., Gansow O.A. and Sjögren H.O. Combination of biotinylation and indium-111 labeling with chelate SCN-Bz-CHX-A-DTPA for chimeric BR96: biodistribution and pharmacokinetic studies in colon carcinoma isografted rats. *Tumor Targeting 1996;2:66-75.*

3. Chen J.Q., Strand S-E. and Sjögren H.O. Optimization of radioiodination and biotinylation of monoclonal antibody chimeric BR96: an indirect labeling using N-succinimidyl-3-(tri-n-butylstannyl)benzoate conjugate. *Cancer Biotherapy & Radiopharmaceuticals 1996;11:217-26.*

4. Chen J.Q., Strand S-E., Isaksson M., Ljunggren K., Sjögreen K., Garkavij M., Tennvall J. and Sjögren H.O. Biodistribution and pharmacokinetics of ^{125}I/^{131}I pair-labeled, biotinylated chimeric BR96 in colon carcinoma isografted rats. *Tumor Targeting 1996:(in press).*

5. Chen J.Q., Strand S-E., Tennvall J., Hindorf C. and Sjögren H.O. Biodistribution and pharmacokinetics of biotinylated ^{188}Re-chiBR96 in colon carcinoma isografted rats. *Submitted to Cancer.*

6. Chen J.Q., Strand S-E., Tennvall J., Lindgren L., Hindorf C. and Sjögren H.O. Extracorporeal immunoadsorption vs. avidin "chase" to enhance tumor-to-normal tissue ratio for biotinylated ^{188}Re-chiBR96. *Revised and re-submitted to Journal of Nuclear Medicine.*

2. ABBREVIATIONS

BADTPA	Bicyclic anhydride diethylenetriaminepenta-acetic acid
BN	Brown Norwegian
CDTAMA	Mono-anhydride trans-1,2-diaminocyclohexane N,N,N′,N′-tetra-acetic acid
ChiBR96	Chimeric BR96
DOTA	1,4,7,10-tetra-azacyclododecane-N,N′,N′′,N′′′-tetra-acetic acid
EC	Electron capture
ECIA	Extracorporeal immunoadsorption (based on the biotin-avidin concept)
HAMA	Human anti-mouse antibody
IFN	Interfron
Ig	Immunoglobulin
Ih	Intrahapatically
IL-2	Interleukin-2
Im	Intramuscularly
IP	Isoelectric point
LET	Linear energy transfer
MAb	Monoclonal antibody
NSTBB	N-Succinimidyl-3-(tri-n-butylstannyl)benzoate
RID	Radioimmunodetection
RIT	Radioimmunotherapy
SCN-Bz-CHX-A-DTPA	2-(p-isothiocyanatobenzyl)-cyclohexyldiethylenetriaminepenta-acetic acid
SPC	N-succinimidyl 5-(tri-alkylstannyl)-3-pyridine carboxylate
TFP MAG_2-GABA	Tetrafluorophenyl mercaptoacetylglycylglycyl-gamma-aminobutyrate
Sr	Subrenal capsule
TCA	Trichloroacetic acid precipitation
TLC	Thin-layer chromatography
T/N	Tumor-to-normal tissue

3. AIMS OF THE STUDY

The purpose of the present study was to investigate suitable radionuclides and labeling methods to enable both radiolabeling and biotinylation of internalizing MAb chiBR96, a prerequisite for using the biotin-avidin concept to enhance tumor/normal tissues (T/N) in radioimmunotherapy (RIT) activity ratios by extracorporeal immunoadsorption (ECIA) or avidin chase. The biodistribution, kinetics and tumor targeting capacity of biotinylated chiBR96 labeled with iodine, indium or rhenium, were evaluated in a colon carcinoma isografted rat model.

THE SPECIFIC AIMS WERE:

1. To evaluate and compare three different chelates for ^{111}In antibody labeling, with respect to *in vivo* stability of the radioimmunoconjugates. The chelates were investigated with indium labeling combined with biotinylation of MAb.

2. To establish a method of iodination and simultaneous biotinylation for chiBR96, rather than electrophilic iodination methods such as Iodogen and Chloramine-T, which were found to be extremely difficult in combination with biotinylation of chiBR96.

3. To achieve rhenium-188 labeling with chelate TFP MAG_2-GABA and simultaneous biotinylation of chiBR96.

4. To compare tumor activity uptake and retention of biotinylated internalizing MAb chiBR96 labeled with ^{125}I, ^{111}In or ^{188}Re in an animal tumor model.

5. To evaluate ECIA with respect to enhancement of the activity ratio of T/N with radiolabeled and biotinylated chiBR96.

6. To compare the T/N radioactivity ratio enhancement achieved with ECIA and avidin chase by using ^{188}Re labeled and biotinylated chiBR96 in the same animal tumor model.

4. INTRODUCTION

4.1 Background for the present study

Monoclonal antibodies (MAbs) which bind to tumor-associated antigens have been used to selectively deliver radionuclides to tumors as a method of tumor therapy, designated radioimmunotherapy (RIT). Radiolabeled MAbs have been effective in eradicating tumor cells *in vitro*, in causing tumor regression in animal tumor models (Canevari et al, 1994) and even in treating hematopoietic malignancies in humans (Kaminski et al, 1996; DeNardo et al 1994; Waldmann et al, 1994). Although RIT has been investigated for nearly two decades, no effective therapeutic procedures have yet been developed for solid tumors (Schreiber et al, 1995). One of the major problems in RIT is the low radioactivity uptake ratio of tumor/normal tissue (T/N) due to the slow systemic clearance of radiolabeled intact MAbs. The high background causes undesired toxicity since normal cells, such as hematopoietic stem cells, are sensitive to the effects of radiation. This limits the amount of activity that can be administered to eliminate all tumor cells (Delalye et al, 1995). Bone marrow reconstitution, transplantation and peripheral blood stem cell harvest, have made it possible to increase the activity of radiolabeled antibodies administered. Toxicity in other organs sensitive to radiation (lung, kidney and liver) might then appear. Press et al have used this approach in treating non-Hodgkin lymphoma with success, but also demonstrated lung toxicity when the absorbed dose exceeded 25 Gy (Press et al, 1995). Strategies, to enhance the radioactivity ratio of T/N by either increasing the tumor uptake and/or decreasing the background, are under extensive investigation (Wilder et al, 1996; Schreiber et al, 1995).

The specificity and extremely high affinity of the biotin-avidin interaction has recently promoted the use of MAb targeting, by manipulating the biodistribution and clearance kinetics of the coupled radioactivity *in vivo* (Schreiber et al, 1995). With radiolabeled and simultaneously biotinylated MAbs, the use of ECIA (based on the biotin-avidin concept) and avidin chase can increase the clearance of blood activity, and consequently enhance the radioactivity ratio of T/N. By using ECIA it might be possible to reduce the activity of radiolabeled MAb in all organs sensitive to radiation (Strand et al, 1989; Strand et al, 1994; Norrgren et al, 1993; Norrgren et al, 1994; Garkavij et al, in press).

Different from non-internalizing MAb, endocytosis and ingestion of internalized MAb in endosomes and lysosomes, following binding to the epitope, enhance the catabolism of the antibody in epitope positive target cells. Localization and redistribution of the radioactive metabolites of the radioimmunoconjugates after being targeted to the tumor cells will significantly interfere with the tumor activity uptake and retention (Fritzberg et al, 1992; Srivastava, 1996).

In the present investigation, three potential therapeutic radionuclides (iodine, indium and rhenium) in RIT and their related labeling methods, have been investigated and compared for the labeling of internalizing MAb chiBR96. Combined with these labeling methods, simultaneous biotinylation of chiBR96 has been developed. The tumor activity uptake and retention could be enhanced by choosing a suitable radionuclide and labeling method. The biodistribution and kinetics of biotinylated chiBR96 radiolabeled with iodine, indium or rhenium, have been studied in a colon carcinoma isografted rat model. The potential use of ECIA to enhance the activity ratio of T/N has also been evaluated and compared with avidin chase.

4.2 Antibodies and their immune responses

4.2.1 Humoral immune system

Acquired immunity is mediated by circulating lymphocytes which recognize antigens specifically by their cell surface receptors. Lymphocytes are mainly grouped into B cells, helper T cells and cytotoxic T cells. When a B cell is activated by recognizing and binding specifically to an exogenous antigen, and is co-stimulated by a correspondingly activated T helper cell, the B cell starts clone expansion, where the clone differentiates to form either a plasma cell which secretes antibodies specific to the antigen, or to a memory B cell which enhances the secondary immune response.

Secreted antibodies (immunoglobulins, Ig) are divided into five major isotypes where IgG, IgE and IgD are monomers, and IgM and IgA are polymers containing a linking peptide of J chain. IgG and IgA are divided into subclasses according to the type of heavy chain. Monomer Ig consists of two identical heavy chains and two identical light chains linked by disulfide bonds. Each heavy chain and light chain has a variable domain on the N terminal, and each variable domain contains a few hypervariable regions that form the antigen binding site of the antibody molecule. Any modification of the hypervariable regions leads to a high

risk of damaging the antibody's antigen-binding capacity. The constant region binds to macrophages and NK (natural killer) cells as well as to the complement system, and through these bindings antibody-dependent cellular toxicity (ADCC) and complement-dependent cellular toxicity (CDC) are mediated.

4.2.2 Monoclonal antibodies

Epitope is the region of an antigen binding to the antibody. An antigen can express several epitopes. Antibodies secreted from plasma cells derived from one B lymphocyte clone are identical or monoclonal, and they all bind to the same epitope. When a tumor antigen is injected into an animal, the B lymphocytes whose surface receptors binding specifically to the epitopes of the antigen are activated. The antibody secreting B lymphocytes are isolated from the immunized animal and fused with immortal myeloma cells in the presence of polyethylene glycol to form the hybridomas. In contrast to the B lymphocytes, the hybridomas can be kept alive for a long time *in vitro* and large amounts of antibodies can be secreted. The anti-tumor MAbs can be obtained by hybridoma cloning and screening of the secreted antibodies. The technique of hybridizing B lymphocyte with myeloma to form MAbs was first reported by Köhler and Milstein (Köhler and Milstein 1975).

4.2.3 Chimeric humanized and human MAbs

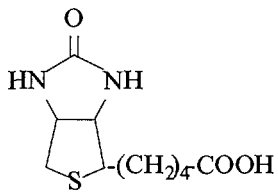
The constant regions of the antibody are mainly responsible for the immune response, or antigenicity. In patient studies with murine MAbs, the human anti-mouse antibody (HAMA) will be created, and the immune complex will be formed when the radioimmunoconjugates are repeatedly administered in radioimmunodetection (RID) and RIT. The use of antibody fragments of $F(ab)_2$ and Fab, obtained by pepsin and papain digestion of intact antibodies, respectively, can minimize the HAMA response, while higher activity accumulation in the kidney and reduced tumor uptake have been reported, hampering their use in RIT (Behr et al, 1996). Humanized and human MAbs can reduce the HAMA response (Weiden et al, 1993).

Chimeric MAb can be obtained by cloning recombinant DNA containing the promoter, leader, and variable region sequences from a mouse antibody gene and the constant region exons from a human antibody gene (Yarnold et al, 1994). The antibody cloned by such a recombinant gene is a mouse-human chimeric MAb. Its antigenic specificity, which is determined by the variable region, is derived from the mouse DNA; its isotype, which is

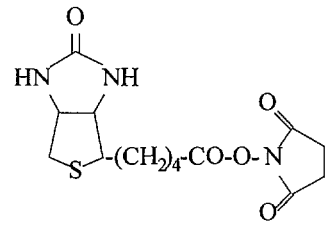
determined by the constant region, is derived from the human DNA. Because the constant regions are encoded by human genes, chimeric MAb has fewer mouse antigenic determinants and is far less immunogenic when administered to humans. Even less murine protein is contained in a reshaped humanized MAb, in which murine hypervariable regions are grafted into human MAb framework regions (Riechmann et al, 1988). A further development is human MAb which has already been generated (James et al, 1987; Ohlin and Borrebaeck, 1996).

4.3 Biotin-avidin/streptavidin and biotinylation

Biotin, with a molecular weight of 244 ($C_{10}H_{16}N_2O_3S$), is water soluble vitamin H, which aroused interest both because of the related deficiency diseases and because of the emerging identification of vitamins as cofactors in various metabolic processes in the 1920s (Richards et al, 1990). The structure of biotin is shown in Figure 1a. Avidin is a 66 kDa glycoprotein, which is commonly isolated from hen egg-white. It shows a strong positive net charge at physiological pH, with an isoelectric point (IP) greater than 10 (Nargessi et al, 1986). Streptavidin, a non-glycosylated analog, is isolated from *Streptomyces avidinii*. In contrast to avidin, streptavidin is nearly neutral at physiological pH, with an IP of approximately 6 (Finn et al, 1985). Each avidin or streptavidin molecule is able to bind up to four biotins with a dissociation constant in the range of 10^{15} M^{-1} (Green et al, 1975).



(a) Biotin



(b) N-hydroxysuccinimido-biotin

Figure 1: The structure of biotin (a) and N-hydroxysuccinimido-biotin (b) a biotin reagent used for protein coupling.

Biotin and avidin/streptavidin have been used in a variety of *in vitro* assays (Wilchek et al, 1984; Wilchek et al, 1990). Recently, it has been under investigation regarding the combination with MAb targeting both preclinically and clinically, due to the availability, ease

of conjugation, and the extremely high affinity and specificity of the biotin-avidin/streptavidin interaction (Schreiber et al, 1995). Because of the small size of the biotin molecule, the biotin-coupling to MAb will not significantly change the biodistribution of the antibody (Wilchek et al, 1990). Conjugation of avidin or streptavidin to a MAb will significantly increase the size, by approximately 40% for an IgG isotype, and may thus alter the pharmacokinetics of the MAb. Avidin/streptavidin also shows an immunogenicity to humans, and patients who have received avidin developed anti-avidin antibodies (Dosio et al, 1993).

Biotin has been coupled to many biochemical compounds such as hormones (Hazum et al, 1986), lipids (Rivnay et al, 1987) and nucleic acids (Herman et al, 1989) for different uses. The conjugation of biotin to MAbs has been thoroughly reviewed (Wilchek et al, 1990). Several biotin linkers that facilitate reactions with proteins are commercially available. Methods of radiolabeling biotin with iodine, indium and yttrium have also been reported (Hnatowich et al, 1987a; Virzi et al, 1991; Ratliff et al, 1995). The average number of biotin molecules coupled to each protein molecule can be determined with enzymatic and radioactive assays (Groman et al, 1990). In the present investigation, the biotin was coupled to the lysine terminal of an antibody by the use of a biotin reagent of N-hydroxysuccinimido-biotin (Figure 1b).

4.4 Choice of radionuclides for RIT and antibody labeling chemistry

4.4.1 The criteria governing radionuclide choice

The criteria applied in the selection of a radionuclide suitable for use in RIT, include its physical and chemical characteristics, ease of production, and the biological factors involved in its *in vivo* biodistribution (Srivastava, 1996). Gamma rays emitted by a radionuclide and which penetrate the body can be used for external scintigraphic imaging to investigate the biodistribution of the radioimmunoconjugates. In contrast, emitted beta particles, which have a short range in tissue and deposit their energy in the vicinity of the decay point, may be used for RIT. Also, the physical half-life of a radionuclide used in RIT should correspond to the biological half-life of the radioimmunoconjugates. Easy MAb coupling with high *in vivo* stability of the radioimmunoconjugates is also important in the choice of radionuclide.

Medium- and high-energy beta particles emitted from radionuclides of ^{67}Cu, ^{177}Lu, ^{131}I, ^{186}Re, ^{188}Re or ^{90}Y can be useful for the treatment of manifest tumors or metastases. These

radionuclides targeted to the antigen-positive tumor cells in the vicinity of antigen-negative tumor cells, which may also be killed by the crossfire effect. For micrometastases and tumor cell clusters, a short-range beta emitter is theoretically preferable, as long-range beta emitters, e.g. ^{188}Re and ^{90}Y, deposit most of their energy outside the target (Howell and Rao, 1989; Strand et al, 1993a).

Table 1: Radionuclides suitable for RIT (Browne et al, 1986; Firestone, 1996; ICRP No. 38, 1983; ICRU No. 37, 1984; ICRP No. 49, 1993)

	Radionuclides	Decay mode (main)	Physical half-life (h)*	Max. particle energy (MeV) (%)	γ energy (keV) (%)	Max range (mm)**
Beta emitters	^{131}I	β^-	8.02 d	0.606 (89.9) 0.334 (7.3) 0.248 (2.1) 0.807 (0.48)	364.5 (81.2)	3.2
	^{90}Y	β^-	64.1	2.28 (99.988)	1760.7 (0.012)	11
	^{186}Re	β^-, EC	90.64	1.07 (74.3) 0.932 (18.8)	137.1 (8.5)	4.6
	^{188}Re	β^-	16.98	2.12 (70.6) 1.97 (26)	155.1 (14.9)	10.1
	^{67}Cu	β^-	61.83	0.392 (57) 0.484 (22) 0.577 (20)	184.6 (48.6)	2.1
	^{153}Sm	β^-	46.27	0.636 (34.7) 0.705 (43.8) 0.808 (20.7)	103.2 (28.3)	3.2
	^{105}Rh	β^-	35.36	0.568 (75) 0.222 (19.7)	319.2 (19)	2
	^{177}Lu	β^-	6.73 d	0.498 (78.6) 0.177 (12.2) 0.385 (9.1)	208.4 (11)	1.7
Alpha emitters	^{212}Bi	α, β^-	60.5 min	6.167 (25.1) α 6.207 (9.8) α 2.254 (55.5) β^-		0.051
	^{211}At	α, EC	7 21	5.982 (41.8) α 7.594 (58.2) α	79.3 (19.3)	0.048
Auger electron emitters	^{111}In	EC	67.32	2.54E-02 (10.6) 5.10E-04 (191)	171.3 (90.2) 245.4 (94)	0.013
	^{77}Br	EC, β^+	57.04	1.23E-02 (0.91) 1.06E-04 (229)	105.8 (13.6)	0.0035
	^{67}Ga	EC	78.27	9.47E-03 (1.31) 4.57E-05 (349)	184.6 (20.4)	0.0024
	^{201}Tl	EC	72.91	7.73E-02 (0.18) 0.27E-03 (174)	70.8 (46) 167.4 (9.4)	0.09
	^{123}I	EC	13.27	3.01E-02 (0.49) 6.99E-04 (180)	159 (83.3)	0.017
	^{124}I	EC, β^+	4.18 d	2.64E-02 (2.48) 6.99E-04 (121)	511 (45.3)	0.015
	^{125}I	EC	59.41 d	3.01E-02 (0.80) 6.99E-04 (299)	27.5 (74)	0.017

* The unit is hours, unless otherwise stated.

** CSDA range in muscle, skeletal.

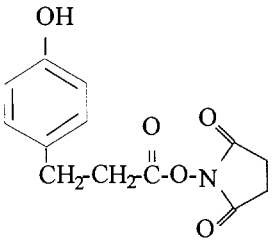
Alpha particles deposit their energy over a much shorter range than beta particles (< 0.1 mm). As yet, alpha-emitting radionuclides have not been used in clinical trials partly because of their short physical half-life and unstable daughter products. Auger electrons as well as low-energy conversion electrons might be useful when the radionuclides such as ^{125}I and ^{111}In (Jönsson et al, 1992; Goddu et al, 1994) are deposited near the critical subcellular components (double strands of DNA), because of their extremely short range (0.4-20 μm) and high LET (Srivastava, 1996). As the demands on antibody specificity in the target are much higher when using short-ranged alpha-emitters or Auger electrons, beta-emitters will probably be the prevailing radioisotopes used in clinical application for a long time to come. Some radionuclides suitable for various types of RIT are listed in Table 1.

4.4.2 MAb iodination

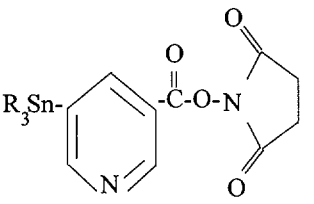
Iodine-131 was selected initially when MAbs were first used and continues to be used for RIT, due to the chemical familiarity, relatively simple labeling, appropriate physical half-life, ready availability and low cost. ^{125}I has also been used in certain circumstances due to its Auger electron emission (Meredith et al, 1995; Bender et al, 1992).

Eclectrophilic iodination methods (direct labeling) were first investigated, and are still used for antibody radioiodine labeling because of their simplicity. The most readily iodinated residues are tyrosine, histidine and cysteine. Under various reaction conditions, e.g. varying pH, different percentages of the tyrosyl, histidyl and cysteinyl moieties have been observed to react with radioiodine, while under physiological pH, i.e. pH around 7.4, the majority of the radioiodine will be coupled to the tyrosine residues (Knight et al, 1978). However, in a process known as deiodination, which is based on the structural similarity between iodinated tyrosine residues and thyroid hormones, iodine can be enzymatically released from the iodine-immunoconjugates iodinated with direct labeling methods. Deiodination of iodinated antibodies is primarily a problem if the targeted antigen undergoes internalization process. Many small molecular conjugates have been investigated for use of MAb indirect iodination to increase the *in vivo* stability of the radioimmunoconjugates. The most commonly employed conjugates for MAb radioiodination are m-, or p-iodobenzoate conjugates. Conjugates of iodobenzoate derivatives, such as N-succinimidyl-3-(tri-n-butylstanny) benzoate (NSTBB), which are structurally different from iodo-tyrosine residues, thyroid hormones and Bolton-Hunter reagent and hence decrease the *in vivo* deiodination, have been extensively

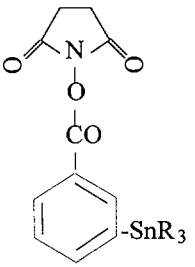
investigated by Zalutsky and other groups (Zalutsky et al,1989; Schuster et al,1991; Wilbur et al, 1989). A thorough review of small molecular conjugates and their coupling chemistry to antibodies has been published (Wilbur et al, 1992). Figure 2 shows examples of the chemical structure of four small molecular conjugates.



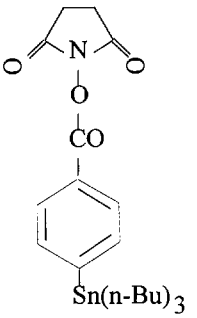
Bolton-Hunter reagent



N-succinimidyl 5-(tri-alkylstannyl)-3-pyridinecarboxylate



N-succinimidyl-3-(tri-alkylstannyl) benzoate



N-succinimidyl-4-(tri-n-butylstannyl) benzoate

Figure 2: Structure of four conjugates as examples of those used for antibody radioiodination (R = methyl or n-butyl).

Although the *in vivo* stability of radioiodinated immunoconjugates is increased by use of the m- or p-iodobenzoate conjugate labeling methods, it may not significantly improve the tumor activity retention when an internalizing MAb is employed, due to the catabolism of the immunoconjugates and the rapid release of the iodobenzonic acid from target cells (Fritzberg and Beaumier, 1992). Conjugates which contain an iodinatable molecule together with a small polysaccharide known to be indigestible and trapped within lysosomes, have recently been investigated. Among them, conjugates of dilactitol-tyramine (DLT) (Strobel et al, 1985) and tyramine-cellobiose (TCB) (Ali et al, 1987) have been studied in more detail. However,

several problems need to be further investigated, such as the low labeling efficiency (Stein et al, 1995) and cross-linking (Ali et al, 1990).

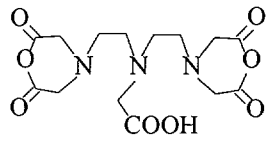
4.4.3 Indium/Yttrium MAb labeling

Activity localization in the reticuloendothelial system due to *in vivo* instability and catabolism of indium-labeled antibodies, and the accumulation of free yttrium in cortical bone, are the main problems in the use of indium/yttrium-labeled immunoconjugates (Arano et al, 1994a; Kinuya et al, 1994). The bicyclic anhydride DTPA was initially used as a chelate agent (Hnatowich et al, 1987b). Recently developed chelates demonstrate increased stability of the ^{111}In-labeled immunoconjugates with decreased accumulation of activity in the liver (Gansow et al, 1990; Camera et al, 1994a; Camera et al, 1994b). Chelates incorporating a cyclohexane ring into the backbone of EDTA or DTPA, such as CDTAMA and SCN-Bz-CHX-A-DTPA, will increase the binding stability to ^{111}In (Fig. 3). The cyclohexane ring locks the nitrogen in the trans-diequatorial configuration and organizes a portion of the ligand into a position favorable for metal chelation (Mease et al, 1989; Srivastava et al, 1991).

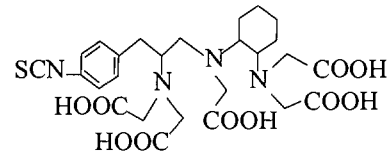
A considerable number of chelates have been investigated regarding ^{111}In/^{90}Y and other radiometal antibody labeling, in which most of them are coupled to the ε-amino group of lysine residues of the antibody. The thiourea bond, through which SCN-Bz-CHX-A-DTPA is coupled to the antibody, is more stable to hydrolysis than the amide bond, formed by one of the carboxylate groups of the ligands of CDTAMA or BADTPA reacting with an ε-amino group of lysine side-chains. Chelate SCN-Bz-CHX-A-DTPA has also been used for yttrium and bismuth antibody labeling with good *in vivo* stability (Camera et al, 1994). Larger chelating ligands (macrocycles) such as DOTA, are extremely attractive in yttrium antibody labeling because of the stability of the radioimmunoconjugates. However, antigenicity, the formation of endogenous antibodies against DOTA (Kosmas et al, 1992), may interfere with the use of the macrocyclic chelate.

With the development of chelate chemistry, ^{90}Y has become an attractive choice for RIT, because of its properties as a beta emitter, high particle energy and sufficiently long physical half-life (2.67 days) for therapeutic use with intact antibodies. The lack of gamma rays for external scintigraphic imaging is one of the disadvantages of ^{90}Y. However, use of bremsstrahlung imaging (^{89}Y) has been reported (Camera et al 1994a). Consequently, ^{111}In-

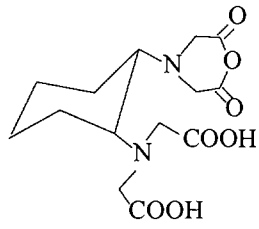
(171 keV and 245 keV gamma rays) labeled antibodies have been used as tracers for ^{90}Y-labeled antibodies, as they have similar biodistributions, and the methods used for ^{111}In antibody labeling can usually be used for ^{90}Y without major modification (Camera, et al 1994; Srivastava, 1996).



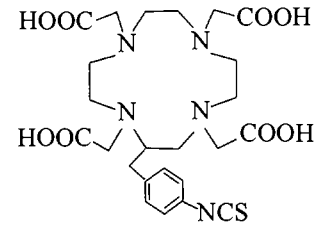
BADTPA



SCN-Bz-CHX-A-DTPA



CDTAMA



SCN-Bz-DOTA

Figure 3: Structure of four chelates as examples of those used for radiometal antibody labeling.

4.4.4 Rhenium MAb *labeling*

^{186}Re and ^{188}Re have recently been used for MAb labeling in RIT (Langmuir et al, 1992). A sufficient abundance (9%) of 137 keV photons in ^{186}Re and 15% 155 keV photons in ^{188}Re allows imaging with a minimal nonspecific absorbed dose. With a chemical similarity to that of technetium, free rhenium will be excreted very rapidly without any accumulation in normal tissues, and decays to stable daughters. The disadvantage of ^{186}Re is that no carrier-free ^{186}Re can be supplied commercially, in which case a significant modification of the immunoconjugates is needed in order to get a specific activity high enough for the therapy. The disadvantage of ^{188}Re is the half-life (17 h), which is too short compared with the biological half-life of most intact antibodies, although ^{188}Re can be obtained from a $^{188}W/^{188}Re$ generator in a carrier-free form, and a half-life of 69 days of the mother radionuclide ^{188}W extends the useful life of the generator to a few months.

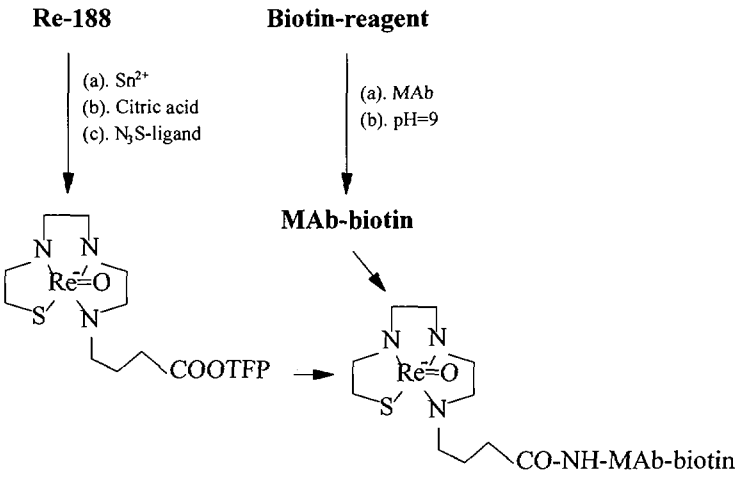


Figure 4: The protocol for rhenium antibody labeling with chelate TFP MAG_2-GABA and simultaneous biotinylation.

The labeling of antibodies with rhenium has to date been carried out according to technetium labeling methods due to their chemical similarities. The chelate method is usually performed by prelabeling the bifunctional chelate with reduced rhenium and then conjugating the complex to the antibodies by the use of carboxylic acid activation prior to reaction with an antibody lysine residue. Several derivatives of diaminodithiol or triaminothiol have been demonstrated as chelates for rhenium antibody labeling (Breitz et al, 1992; Visser et al, 1993; Ram et al, 1994), in which the chelate TFP MAG_2-GABA has been routinely used for clinical trials (Weiden et al, 1993; Breitz et al, 1993; Breitz et al, 1995a) as well as for our MAb labeling and simultaneous biotinylation (Figure 4). Direct rhenium labeling has also been investigated (Griffiths et al, 1991; Griffiths et al, 1994), although it is believed that it will be difficult to use in clinical RIT studies due to the *in vivo* instability (Su et al, 1992; Visser et al, 1993).

4.4.5 Other radionuclides for MAb labeling

Besides iodine, conjugates of m- or p-iodobenzoate have also been used for other halogens such as bromine and astatine antibody labeling (Zalutsky et al, 1994; Larsen et al, 1994; Wilbur, 1992). The chelate of SCN-CHX-Bz-A-DTPA has been used for alpha-emitter ^{212}Bi MAb labeling with high *in vivo* stability (Hartmann et al, 1994). ^{67}Co has been used for MAb labeling with the macrocyclic chelate DOTA (Moi et al, 1988). The macrocyclic bifunctional

chelating agent DOTA can also increase the stability of radioimmunoconjugates labeled with ^{153}Sm as well as other lanthanides (Srivastava, 1996; Cacheris et al, 1987).

4.5 Strategies to improve the efficacy of RIT

Although the use of an iodinated antibody in tumor visualization was demonstrated as early as 1953 (Pressman and Korngold, 1953), and the first clinical therapeutic trial with ^{131}I-labeled polyclonal antibodies was conducted in the 1960s (Spar et al, 1967), RIT is a relatively new field. Only around one hundred radioimmunotherapy clinical trials have been reported during the last two decades (Wilder et al, 1996). Problems that currently face RIT include circulating free antigen, binding of antibodies to nonspecific Fc receptors, insufficient tumor penetration, antigenic heterogeneity, insufficient antigen expression and development of HAMA, since the majority of the antibodies used in RIT so far are of murine origin. All of these problems result in low tumor targeting, relatively high activity accumulation in normal tissues and consequently a low T/N activity ratio (Buchsbaum et al, 1995).

Strategies combined with RIT, either in animal or in clinical studies, to enhance the therapeutic efficacy, are summarized and listed in Table 2. The combination with cytokines, for example interferons (IFN) increases the expression of tumor-associated antigens, and interleukin-2 (IL-2) improves the permeability of the antibodies into tumor tissue.

The use of antibody fragments improves the T/N activity ratio as well as the early contrast of RID, but the fast systemic clearance limits sufficient activity accumulation in the tumor tissue, resulting in a lower tumor uptake than with the intact antibody (Stein et al, 1994).

In vivo stability of radiometal-labeled immunoconjugates used in RIT is important, because the release of the activity from the immunoconjugates will decrease the targeting in the tumor and the free radiometal as well as the other radioactive metabolites are usually accumulated in normal tissues. A concept that has been investigated to increase the activity ratio of T/N for radiometal MAb labeling, is a chemical modification by the insertion of a labile linkage between antibody and chelating ligand, which will be cleaved from the antibody by normal tissue metabolism (Quadri et al, 1993; Arano et al, 1994b). The ligand-bound activity will then be excreted rapidly from the whole body.

Table 2: Strategies combined with RIT to enhance the therapeutic efficacy in preclinical and clinical studies.

To increase activity uptake in tumor	1. Optimal choice of radionuclide and labeling method (Srivastava, 1996) 2. Pre-load with cold antibodies (Garkavij et al, 1994; Kaminski et al, 1996a). 3. Combined use with cytokines such as IFN-α and IFN-γ (Murray et al, 1995; Greiner et al, 1993), and IL-2 (DeNardo et al, 1991). 4. External-beam radiation therapy (Msirikale et al, 1987) 5. Local hyperthermia (Wilder et al, 1993). 6. Local electropermeabilization (Persson et al, 1992; Strand et al, 1995).
To provide synergism for tumor cell killing	7. External-beam radiation therapy (Amin et al, 1995). 8. Chemotherapy (Chalandon et al, 1992). 9. Hypoxic cytotoxins (Wilder et al, 1994).
To decrease normal tissue toxicity	10. Antibody fragments (Juweid et al, 1996). 11. Pre-targeting (Breitz H et al, 1995). 12. High activity combined with autologous bone marrow transplantation (Kaminski et al, 1996b). 13. Avidin chase (Paganelli et al, 1994) 14. ECIA (Strand et al, 1994).
To reduce HAMA	15. Humanized or human antibodies (Ohlin and Borrebaeck, 1996).

Pre-targeting, for example two- or three-step approaches, most of which are based on the biotin-avidin system, have been extensively investigated for RID and RIT because of the high affinity and specificity of the biotin-avidin interaction (Hnatowich et al, 1987; Zhengsheng et al, 1995). T/N radioactivity ratios have been increased in animal tumor models (Khawli et al, 1993; Saga et al, 1994) as well as clinical trials (Paganelli et al, 1991; Dosio et al, 1993). The two-step approach is briefly described as follows. Biotin- or avidin/streptavidin-coupled MAb is first targeted in the tumor cells; after the circular immunoconjugates have been excreted, the radiolabeled avidin/streptavidin or biotin is administered. The three-step approach has been thoroughly investigated both in animal tumor models (Axworthy et al, 1996) and in clinical studies (Breitz et al, 1995a). After the pre-targeting of streptavidin-coupled MAb and the removal of circulating immunoconjugates by the clearing agent of biotin-HSA (human serum albumin), the ^{90}Y-DOTA-biotin is finally administered. More than 90% of the radioactivity can be excreted from the whole body within 24 h post-injection, with a

maintained high tumor uptake (Axworthy et al, 1996; Breitz et al, 1995b). However, although two- and three-step approaches are under investigation and have shown promising results, they can hardly be used for internalizing MAbs.

Other approaches, also based on the biotin-avidin system, are ECIA (Strand et al, 1989; Strand et al, 1994; Norrgren et al, 1992; Garkavij et al, 1993) and avidin chase (Marshall et al, 1994; Kobayashi et al, 1994). These strategies require the MAb to be radiolabeled and simultaneously biotinylated. Both preclinical and clinical studies have been performed (DeNardo et al, 1993; Paganelli et al, 1994). In the avidin chase approach, activity in the circulation is removed by the injection of avidin or streptavidin resulting in the formation of an avidin-biotin-MAb complex, and fast accumulation and catabolism of the complex in the liver tissue.

4.6 Extracorporeal immunoadsorption

Extracorporeal immunoadsorption (ECIA) (Strand et al, 1989) was derived from plasmapheresis, which is used clinically for the removal of antibodies in autoimmune diseases. Circulating radiolabeled immunoconjugates are removed by passing the blood through an absorption column. Sgouros (1992) demonstrated the feasibility of combining plasmapheresis with high-dose administration of radiolabeled MAb in order to overcome the so called "binding-site" barrier. Subsequent removal of unbound circulating MAbs in the blood by ECIA would facilitate a reduction of the absorbed dose to whole body and radiosensitive tissues, i.e. bone marrow, lung and liver.

Based on the biotin-avidin reaction, ECIA has been extensively investigated by the Lund group. We have investigated three different kinds of MAbs, which were both radioiodinated and biotinylated, together with an avidin-gel absorption column in different animal tumor models (Strand et al, 1994; Norrgren et al, 1993; Norrgren et al, 1994; Garkavij et al, 1993; Garkavij et al, in press). Rats were catheterized with an arterial (aorta carotis) and a vein (vena jugularis) catheter to gain blood access. ECIA was performed 2-3 days after the catheterization. Blood was pumped from the arterial catheter, passed through an absorption column at a flow rate of 1.5 ml/min, and then returned by the venous catheter to the rat. The ECIA procedure was completed within 2 hours. During this period more than three blood volumes had passed through the avidin-absorption column, which contained 1.2 ml of avidin-sepharose 6MB gel with a concentration of avidin of 2 mg/ml gel. Before being connected to

the ECIA system, the column was rinsed with 0.9% sodium chloride in order to clean the column from any traces of free avidin. A detailed description of our ECIA system can be found elsewhere (Norrgren et al, 1993; Garkavij et al,1996).

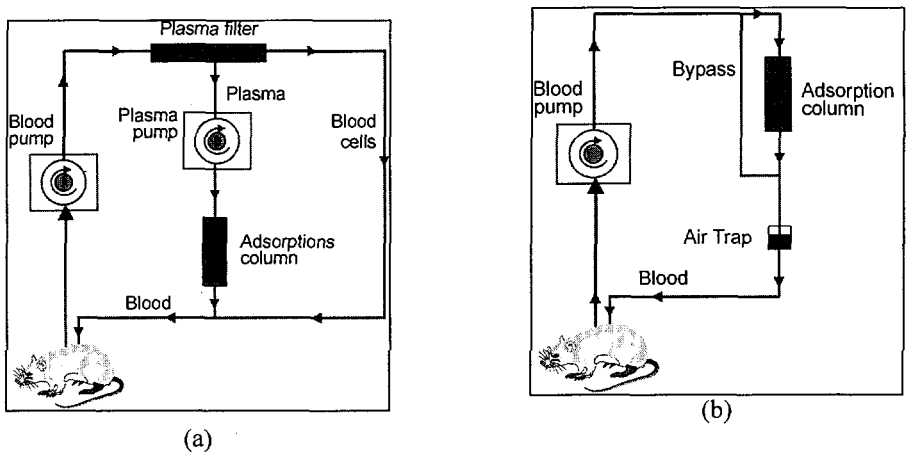


Figure 5: Schematic of (a) ECIA of plasma separated with a plasma filter and (b) ECIA of whole blood.

The starting time for ECIA was determined from previous biokinetic studies of each biotinylated and simultaneously radioiodinated MAb, and was dependent on the activity distribution in the tumor, blood and normal tissues. Circulating unbound radioimmunoconjugates were removed by ECIA without significant change in the tumor uptake. The uptake ratio of tumor-to-normal tissue was increased in blood-rich and radiosensitive normal tissues, such as liver, bone marrow and kidney (Strand et al, 1994).

Other groups have used ECIA in a few clinical cases and shown a reduced toxicity (Lear et al, 1991; Dienhart et al, 1994). Anti-antibody adsorption columns were employed in these studies, which implies a specific column for each antibody used.

5. MATERIALS AND METHODS

BADTPA and the biotin reagent N-hydroxysuccinimido-biotin were purchased from Sigma. CDTAMA was received as a gift from Suresh C. Srivastava, Brookhaven National Laboratory, USA, SCN-Bz-CHX-A-DTPA from Otto A. Gansow, NIH, Bethesda, USA, and TFP MAG_2-GABA from NeoRx Co., Seattle, USA.

5.1 NSTBB synthesis

The conjugate of NSTBB (Fig. 2) was synthesized according to the method developed by Zalutsky with a slight modification (Zalutsky et al, 1987). Briefly, in a 100 ml three-necked bottle, 1.25 g m-bromobenzoic acid dissolved in 35 ml of dry tetrahydrofuran (THF) was maintained under nitrogen gas, magnetic stirring conditions and kept in an ether-dry ice-liquid nitrogen bath. When the temperature reached -100°C, 8 ml of 1.6 M n-butyl lithium in hexane was added slowly during 20 min and kept at a temperature below -95°C. The mixture was then warmed to -75°C for 30 min and quenched by the addition of 4.15 g tri-n-butyl tin chloride in 10 ml dry THF. After another 30 min incubation at -75°C, the reaction mixture was warmed to room temperature and stirred for 1.5 h. After diluting the reaction solution with 100 ml H_2O, the organic phase was extracted four times with 80 ml ether, washed with 5% aqueous NaOH and then with water until the aqueous extract became neutral. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed using a rotary evaporator, and an organic compound was obtained. Six hundred ninety milligrams of N-hydroxysuccinimide (NHS) and 1.24 g of dicyclohexylcarbodiimide (DCC) together with 20 ml of dry THF were added and mixed with the obtained organic compound. The mixture was kept under nitrogen gas and stirred overnight at room temperature. The reaction mixture was filtered to remove precipitated dicyclohexylurea, and the solvent was evaporated using a rotary evaporator. NSTBB reagent was isolated by flash chromatography over a silica gel column (20-200 mm), first eluted with 300 ml 10% ethyl acetate/hexane, then eluted with 300 ml 20% ethyl acetate/hexane which was collected and dried using a rotary evaporator. The purified NSTBB was identified by ^1H nuclear magnetic resonance.

5.2 Monoclonal antibodies

Mouse-human chimeric BR96 (chiBR96) (Bristol-Myers Squibb) is a human IgG1 isotype of murine BR96 (Hellström et al, 1990), and binds to a tumor-associated cell surface antigen

Lewis-Y. ChiBR96 is rapidly internalized after binding to the epitope (Yarnold et al, 1994). According to immunohistology, BR96 binds to the majority (>75%) of human carcinomas of colon, lung, breast, ovary, stomach, pancreas, esophagus and cervix (Hellström et al., 1990). As with all the other tumor-associated MAbs so far described, it is not completely tumor specific, but also reacts with some normal tissues of humans, rat, dog and monkey. However, only a limited number of normal human tissues express the BR96 antigen, such as differentiated cells within the gastrointestinal epithelium and the acinar cells of the pancreas. It has been demonstrated that BR96 is an oxidant sensitive antibody, and that it could be inactivated even under mild oxidation conditions (Abraham et al, 1991). Drug-chiBR96 immunoconjugates have also been used for solid tumor therapy, resulting in complete regression and cure of xenografted human lung, breast and colon carcinomas growing subcutaneously in athymic mice (Trail et al, 1993).

The monoclonal antibodies L6 (DeNardo et al, 1994b) and p256 (Stuttle et al, 1989) have also been used for the stability determination of ^{111}In labeling (Paper I).

5.3 Animal tumor models

Brown Norwegian (BN) rats or BN crossed with Wistar Fourth rats, which express chiBR96 epitope in normal tissues such as pancreas and gastrointestinal tract, have been used in the present investigation. BN 7005 rat colon carcinoma expressing chiBR96 epitope was originally induced by 1,2-dimethylhydrazine. Immunocompetent BN male rats bearing isografts of BN 7005 colon carcinomas were used to avoid the limitations in human tumor xenografted immunodeficient animal models, and as the results are more easily transferred to the human situation (Canevari et al, 1994; Dincalci, 1994). The stem tumor serially passaged in BN rats, was homogenized together with the same volume of buffer solution and used for tumor inoculation. Fifty microliters of tumor suspension (a ratio of 1:8 for tumor homogenized solution to buffer solution at a final ratio of 1:16 for tumor to buffer in volume) were inoculated intramuscularly (im), or beneath the subrenal capsule (sr) or intrahepatically (ih). BN rats carrying (im) and (sr) tumors were used for *in vivo* investigation of iodinated and biotinylated chiBR96 (Papers III-IV). BN rats with (im), (sr) or (ih) tumors were used to study biotinylated ^{111}In-chiBR96 (Papers I-II), and rats carrying (im), (sr) and (ih) tumors were used for *in vivo* investigations of biotinylated ^{188}Re-chiBR96 (Papers V-VI).

Nude athymic rats carrying (im) and (sr) xenografts of H2981 human lung carcinoma, were also used in the first study (Paper I).

5.4 Biotinylation and simultaneous radiolabeling

N-hydroxysuccinimido-biotin was used for the biotinylation of antibodies in the present investigation. Under certain circumstances, e.g. concentration of the antibody and pH, a series of biotinylation levels (5-60 μg biotin reagent per mg antibody, corresponding to 2.2-26.4 molar ratios of biotin to added antibody) was investigated with respect of obtaining high enough avidin-gel binding capacity without significantly interfering with the antigen binding capacity of the biotinylated MAb. The number of biotin molecules coupled to each antibody was determined by the use of 4′-hydroxyazobenzene-2-carboxylic acid (HABA) dye assay (Green et al, 1965). Under optimal conditions, about 2-3 biotin molecules were coupled to each antibody, which was then combined with iodine, indium or rhenium radiolabeling.

5.4.1 Combination of iodine labeling and biotin coupling

The method of iodination and simultaneous biotinylation for chiBR96 was established, and is described in detail in Paper III. BR96 is an oxidant sensitive MAb, and our previous radioiodine labeling experiments demonstrated that electrophilic iodination methods were particularly difficult to use in combination with biotinylation for chiBR96 (Abraham et al, 1991; Paper III). The antigen binding capacity of antibodies was destroyed even with a single biotin coupled to each of the iodinated chiBR96 labeled with Iodogen or Chloramine-T methods, although the non-biotinylated ^{125}I-chiBR96 can preserve the immunoreactivity (Paper III).

In contrast to electrophilic iodination methods, the iodinated conjugate of NSTBB as well as the biotin reagent are coupled to the ε-amino groups of lysine residues in the antibody. Iodination using the conjugate NSTBB can avoid the modification of tyrosine residues, which has a high probability of being involved in the hypervariable regions of the antibody (Nikula et al, 1995), and avoid oxidation of the antibody, which are problematic when electrophilic labeling methods are used. The whole procedure of the iodination and biotinylation can be completed within 2 hours with an approximately 40% radiochemical yield.

The biodistribution and antigen targeting capacity of the biotinylated ^{125}I-chiBR96 were evaluated and compared with those of the non-biotinylated ^{125}I-chiBR96, both of them were iodinated with the NSTBB conjugate (Paper III). A direct comparison of tumor targeting capacity and activity distribution was also performed under identical circumstances by investigating each animal after the administration of a mixture of biotinylated ^{125}I-chiBR96 (NSTBB labeling method) and non-biotinylated ^{131}I-chiBR96 (Iodogen labeling method) Paper IV).

5.4.2 Combination of indium labeling and biotin coupling

Indium antibody labeling was performed according to the methods described by Mirzadeh (Mirzadeh et al, 1990). Three different chelates, BADTPA, CDTAMA and SCN-Bz-CHX-A-DTPA were evaluated and compared regarding antibody indium labeling, as well as for the biotinylation combination with respect to the stability of the radioimmunoconjugates (Paper I). Biotinylated ^{111}In-chiBR96 labeled using the chelate SCN-Bz-CHX-A-DTPA was further investigated (Paper II). The labeling and simultaneous biotinylation procedure, with slight modifications for the three different chelates (Paper III), briefly includes: first, coupling of the biotin reagent and chelate to the lysine residues of the antibody; then, separation of the biotin- and chelate-coupled antibodies from extra free chelate and biotin reagent, and pH adjustment to about 4.5 using small column centrifugation; finally, binding of indium to the immunoconjugates. Although all the three chelates are coupled to ε-amino groups of lysine in the antibody, different bonds were formed with different *in vivo* stability against hydrolysis (for details see INTRODUCTION).

The *in vivo* stability and tumor targeting capacity of the ^{111}In-chiBR96, labeled with chelate agents of BADTPA, CDTAMA or SCN-Bz-CHX-A-DTPA, have been compared according to the activity accumulation in liver and tumor uptake, respectively. The kinetics of biotinylated ^{111}In-chiBR96 labeled with SCN-Bz-CHX-A-DTPA has been further investigated up to 8 days after the antibody administration. Activity uptake and retention in the tumor tissues as well as the activity distribution in the normal tissues of interest for the biotinylated ^{111}In-chiBR96 have been compared with those of the biotinylated ^{125}I-chiBR96 labeled with the NSTBB conjugate method (Chen et al, 1995; Chen et al, manuscript).

5.4.3 Combination of rhenium labeling and biotin coupling

For the combination of biotin coupling and ^{188}Re labeling, MAb chiBR96 was biotinylated and purified from free biotin reagent prior the ^{188}Re labeling (Paper V). The ^{188}Re labeling procedure was modified somewhat according to the method established by Fritzberg (Fritzberg et al, 1988; Breitz et al, 1992), because of the relatively large volume of ^{188}Re eluted from ^{188}W/^{188}Re generator. As the concentrations of the chelate and antibody will interfere with the efficiency of the labeling, an optimal condition of 1 : 10 (v/v) of 4.2 mg/ml $SnCl_2$ solution to fresh ^{188}Re elution with chelate TFP MAG_2-GABA at a molar ratio to 1-1.5 of the antibody, was used after having determined a series of different amounts of the stannous ion and chelate (Paper V). Bovine serum albumin (BSA) and ascorbic acid were added to the purified biotinylated ^{188}Re-chiBR96, giving final concentrations of 1 mg/ml and 5 mg/ml respectively, to protect against the radiolytic effect, and to ensure that the ^{188}Re-labeled immunoconjugates would remain intact until administration.

5.4.4 Biodistribution and tumor targeting capacity: comparison of radiolabeled chiBR96 with and without biotinylation

As well as the *in vitro* target cell binding analysis, the biodistribution and tumor targeting capacity of iodine, indium and rhenium radiolabeled chiBR96 with and without biotinylation were compared in the animal tumor model, to determine whether the stability and immunoreactivity of the immunoconjugates had changed or not, as a result of the biotinylation procedure of the antibody. The stability of the radioimmunoconjugates could be determined as that the instability of the ^{125}I-chiBR96 causes high activity accumulation in the thyroid and small intestine, and high activity accumulation in the liver/spleen usually occurs due to the instability of ^{111}In-chiBR96. Both instability and low immunoreactivity of the immunoconjugates could result in low tumor activity uptake. The comparison was performed at 24 and 48 hours post-injection for ^{125}I-, ^{111}In- and ^{188}Re-labeled chiBR96.

5.5 Quality control

The techniques used for quality control in the present investigation are thin-layer chromatography (TLC), trichloroacetic acid precipitation (TCA), size exclusion chromatography and Protein-A affinity chromatography. Also, the efficiency of the iodine binding to the conjugate of NSTBB and the efficiency of rhenium binding to the chelate of

TFP MAG_2-GABA, were evaluated by TLC developed with different mobile phases and combined with UV lamp detection and/or scanner with a 1 mm slit-collimated NaI(Tl) crystal (5 mm thick, 50.8 mm diameter) with a beryllium window (Persson and Darte, 1974) (Papers III and V).

Radiolabeled immunoconjugates used for *in vitro* and *in vivo* studies have a 97% or higher radiochemical purity for iodine- and indium-labeled antibodies, and a 95% or higher radiochemical purity for ^{188}Re-labeled antibodies. The radiochemical purity of the radiolabeled immunoconjugates in the sera at different intervals post-injection have also been determined.

5.5.1 In vitro and in vivo stability determination

The stability of the ^{111}In-MAbs in *in vitro* serum incubation or *in vivo* plasma samples can be easily determined by high pressure liquid chromatography (HPLC), because most of the free indium will be bound by albumin or transferrin with different retention times from that of ^{111}In-coupled immunoconjugates. In Paper I, a method was demonstrated to determine the relative stability of ^{111}In-labeled antibodies by TCA precipitation after serum incubation together with DTPA at a 5×10^3 molar excess to the antibodies added. Although artificial results would probably be obtained under acidic conditions, e.g. an increase in the release of indium from the chelates, the stability of the ^{111}In-labeled immunoconjugates with different chelates can be compared. The *in vivo* stability of the ^{111}In-labeled L6, IgG_{2a}, was determined by analyzing the serum samples obtained at different intervals post-injection with protein-A affinity chromatography.

The stability of iodine- and rhenium-labeled immunoconjugates was analyzed by TCA precipitation, and by TLC with a mobile solution of 10% aqueous ammonium acetate : methanol (1 : 1), or 12% TCA.

The stability of the biotinylation was determined by checking the binding capacity of the biotin-coupled immunoconjugates to the avidin-agarose gel (Agarose-avidin, Vector, Burlingame, USA). The binding capacity was calculated as a percentage of the avidin-agarose gel binding activity of the total added activity.

5.5.2 Immunoreactivity determination

The immunoreactivity of radiolabeled chiBR96, and simultaneously biotinylated and radiolabeled chiBR96 was analyzed using BN 7005 rat colon carcinoma cells as target cells. The binding capacity of the immunoconjugates to the targeted cell was determined before administration, and compared with that of the *in vivo* plasma samples obtained at different intervals up to 48 hours post-injection (Papers II-III).

5.6 Biodistribution and kinetics

Eight male nude athymic rats weighing 235 ± 45 g, bearing human lung carcinoma H2981 cells intramuscularly (im) and beneath the subrenal capsule (sr) were used for the *in vivo* studies of ^{111}In-labeled MAb L6. One hundred fifty-eight BN rats carrying isografts of BN 7005 rat colon carcinomas (im), (sr) and/or (ih) were used for *in vivo* stability, biodistribution and kinetic studies of radiolabeled chiBR96 with and without biotinylation. Fifty micrograms of ^{111}In-L6, and 50-100 μg of radiolabeled chiBR96, with a specific activity of 63-91, 35-52 or 110-130 kBq per μg of antibody for ^{125}I, ^{111}In or ^{188}Re, respectively, were administered. The kinetics was investigated at regular intervals up to 192 h post-injection for ^{125}I- and ^{111}In-labeled chiBR96, while a 72 hour investigation period was used for ^{188}Re-chiBR96 because of its short physical half-life. The activity uptake in tumors and normal tissues of interest were expressed as a percentage of the injected activity per gram tissue (%/g). The activity distributions were investigated for the biotinylated chiBR96 radiolabeled with iodine, indium and rhenium, as well as with related different labeling methods. Especially, the activity uptake and retention in the tumor tissue were compared for biotinylated chiBR96 simultaneously labeled with ^{125}I, ^{111}In or ^{188}Re.

5.7 Scintillation camera imaging

Whole-body imaging was performed using a scintillation camera (General Electric 400T, GE, Milwaukee, WI, USA). The use of collimator (low- or medium-energy collimators) and width of the energy windows for these radionuclides (^{125}I, ^{131}I, ^{111}In or ^{188}Re) were chosen according to the spatial resolution and counting sensitivity. The imaging was carried out immediately after the antibody injection (before antibody catabolism, and was regarded as total administrated activity), and at regular intervals. Images were stored in a computer with Gamma-11 software for the analysis. The total number of counts in a region-of-interest

including the entire animal was obtained from the images. After radioactive decay correction and background subtraction, the counts were used for the calculation of activity retention in the body. Imaging was also used for tumor visualization.

5.8 Beta camera imaging

Beta-camera imaging was used to determine the activity distribution within the tumor tissue for the iodine radiolabeled and biotinylated chiBR96 at 48 h post-injection (Paper IV). Details on the "beta-camera" can be found elsewhere (Ljunggren et al, 1993).

5.9 ECIA and avidin chase

Based on the biodistribution and kinetics, ECIA has been investigated (Garkavij et al, in press) for biotinylated ^{125}I-chiBR96, with the NSTBB iodination method described in Papers III-IV. The results showed that 85% of the plasma activity could be removed by the ECIA procedure. The T/N activity ratio was enhanced 2.3-3.5 fold for blood-rich normal tissues, such as liver, lung, kidney and bone marrow, compared with that of the controls (Garkavij et al, in press).

Based on the investigations of the kinetics of the biotinylated ^{188}Re-chiBR96 (Paper V), ECIA was performed at 6 or 12 h post-injection. Activity redistribution was investigated just after the ECIA (8 or 14 h post-injection, respectively) and 40 or 34 h after this procedure (i.e. 48 h post-injection).

With radiolabeled and simultaneously biotinylated MAb, the avidin chase approach could also be used to improve the tumor-to-blood ratio for internalizing MAbs, such as chiBR96. Both ECIA and avidin chase have been separately explored both preclinically (Norrgren et al, 1992; Marshall et al, 1994) and clinically (Dienhart et al, 1994; Paganelli et al, 1994; Paganelli et al, 1990). A direct comparison between these two strategies has for the first time been carried out with respect to T/N activity ratio enhancement for radioimmunoconjugates in the same animal model in the present investigation (Paper VI).

5.10 Statistical analysis

The rate of radioactivity clearance from the whole body and blood was calculated by least-squares fit using the computer program BMDP (Dixon et al, 1990). Statistical analysis was performed using Student's t test for unpaired data.

6. RESULTS AND COMMENTS

6.1 ^{111}In MAb labeling with three different chelates

Chelate CDTAMA has been used in indium labeling for MAbs 17-1A (Mease et al, 1990) and L6 (Paper I), resulting in an enhanced *in vivo* stability and higher tumor uptake, compared with the use of chelate BADTPA. For MAb chiBR96 ^{111}In labeling with CDTAMA, a higher activity accumulation in liver occurred, and the tumor uptake was lower than that of ^{125}I-chiBR96. Our previous results prompted us to establish a method for chiBR96 ^{111}In labeling with a more stable chelate. The chelate SCN-CHX-Bz-A-DTPA, a recently developed backbone-substituted derivative of DTPA has shown good *in vivo* stability in indium, yttrium as well as alpha-emitter bismuth MAb labelling (Camera et al, 1993; Tiffany et al, 1994), was chosen for chiBR96 indium labelling. ^{111}In-chiBR96 using SCN-CHX-Bz-A-DTPA demonstrated a low liver uptake with an improved tumor uptake. Figure 5 shows the activity uptakes in tumor, liver and blood of biotinylated ^{111}In-chiBR96 labeled using one of these three different chelates. All three ^{111}In-labeled immunoconjugates had the same level of biotinylation and chelate coupling.

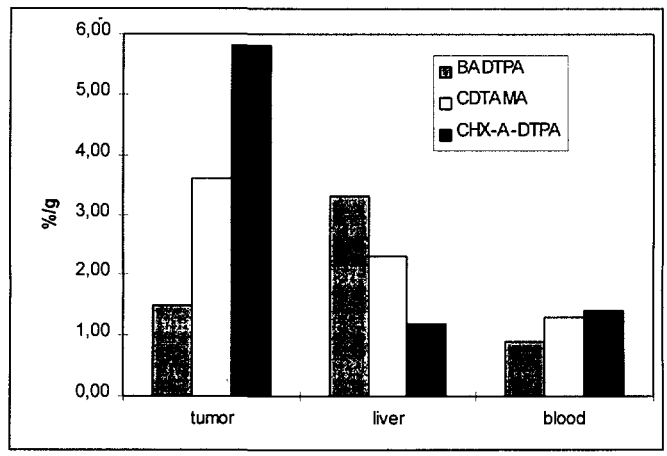


Figure 5: Activity uptakes in tumor, liver and blood of biotinylated ^{111}In-chiBR96 labeled with one of three different chelates (BADTPA, CDTAMA or SCN-CHX-Bz-A-DTPA) in colon carcinoma isografted BN rats at 24 hours post-injection.

6.2 Iodine-chiBR96 labeled with NSTBB or Iodogen methods

The Iodogen method has been used for chiBR96 iodination without significantly changing the antigen binding capacity of the antibody, but it was particularly difficult in combination with biotinylation. Iodination using the conjugate NSTBB has been successfully introduced into the simultaneous iodine radiolabeling and biotinylation of chiBR96 (Paper III). A comparison of biodistribution and kinetics between biotinylated ^{125}I-chiBR96 using NSTBB conjugate and non-biotinylated ^{131}I-chiBR96 labeled with the Iodogen method, has been carried out under identical circumstances. The tumor uptake of biotinylated ^{125}I-chiBR96 was significantly higher than that of ^{131}I-chiBR96 up to 96 h post-injection, but with the same kinetic (Paper IV, Fig. 2). Thyroid activity uptake of biotinylated ^{125}I-chiBR96 was significantly lower than that of ^{131}I-chiBR96. The thyroid ^{131}I uptake was 6 times higher than that of ^{125}I at 8 days post-injection. Activity accumulation of ^{131}I-chiBR96 in the stomach was also significantly higher than that of the biotinylated ^{125}I-chiBR96 (Table 3). Kinetic analysis demonstrated that the whole-body activity retention varied between these two iodination methods. Activity of ^{131}I-chiBR96 was cleared from the whole body with a half-life of 86.6 h, while the activity of biotinylated ^{125}I-chiBR96 was eliminated from body with a short half-life ($T_{1/2\alpha}$) of 5.9 h and a long half-life ($T_{1/2\beta}$) of 72.8 h, corresponding to 12% and 88% of the administered activity, respectively.

Table 3: Comparison of activity distribution of biotinylated ^{125}I-chiBR96 (NSTBB conjugate labeling method) and non-biotinylated ^{131}I-chiBR96 (Iodogen labeling method) in colon carcinoma isografted BN rats at different times post-injection (%/g) (n=6) [mean(1SD)].

	8 h		24 h		48 h		96 h		192 h	
	NSTBB	Iodogen	NSTBB	Iodogen	NSTBB	Idogen	NSTBB	Iodogen	NSTBB	Iodogen
Tumor (im)	2.0(0.4)	1.6(0.4) *	2.2(0.2)	1.6(0.5) *	2.3(0.4)	1.9(0.3) *	1.4(0.3)	0.9(0.2) **	0.4(0.1)	0.3(0.1)
Tumor (sr)	4.0(0.5)	3.2(0.5) *	4.1(0.6)	2.8(0.5) **	3.1(0.4)	2.5(0.4) *	1.7(0.4)	1.1(0.2) **	0.2(0.1)	0.2(0.1)
Blood	2.6(0.2)	2.4(0.2)	1.5(0.3)	1.5(0.5)	0.9(0.1)	1.1(0.1)	0.4(0.1)	0.5(0.1)	0.1(0.1)	0.1(0.1)
Kidney	0.9(0.1)	0.8(0.1)	0.5(0.1)	0.5(0.0)	0.3(0.0)	0.3(0.0)	0.2(0.0)	0.2(0.0)	0.03(0.0)	0.05(0.0)
Liver	1.1(0.1)	0.9(0.2)	0.7(0.3)	0.4(0.1) *	0.5(0.2)	0.4(0.1)	0.2(0.1)	0.2(0.0)	0.06(0.0)	0.05(0.0)
Stomach	0.4(0.2)	0.5(0.2)	0.2(0.1)	0.4(0.1) **	0.1(0.0)	0.3(0.1) ***	0.1(0.0)	0.2(0.1) ***	0.02(0.0)	0.10(0.0) ***
Lung	1.0(0.1)	0.9(0.1)	0.6(0.2)	0.6(0.1)	0.4(0.1)	0.4(0.1)	0.1(0.0)	0.2(0.0)	0.03(0.0)	0.06(0.0)
Thyroid‡	0.3(0.1)	0.5(0.1) **	0.5(0.3)	1.9(0.7) ***	1.0(0.3)	2.9(1.0) ***	1.0(0.6)	3.9(0.7) ***	0.7(0.2)	3.7(0.2) ***

*0.05<P>0.01, **0.01<P>0.001, ***P<0.001; ‡ % of the administered activity.

6.3 ^{188}Re labeling and simultaneous biotinylation

Under optimal labeling conditions, a 35-40% radiochemical yield was obtained with 60-70% chelate-^{188}Re binding and about a 60% chelate-^{188}Re antibody coupling efficiency. Simultaneous biotinylation of chiBR96 using 18 μg/mg antibody N-hydroxysuccinimido-biotin, incubated for 1 hour at room temperature, resulted in about 2 biotin molecules being coupled to each antibody with a 79-92% avidin-gel binding capacity. The antigen binding capacity of biotinylated ^{188}Re-chiBR96 was not significantly changed compared with that of the ^{188}Re-chiBR96. The biodistribution of the biotinylated ^{188}Re-chiBR96 (Table 4) was similar to that of the biotinylated ^{125}I-chiBR96.

Table 4: Biodistribution of the biotinylated ^{188}Re-chiBR96 in tumors and some normal tissues of interest at different times post-injection in colon carcinoma isografted BN rats (%/g) (mean ± SD) (n = 6)

	15 min*	1 h	6 h	12 h	24 h	48 h	72 h
Tumor(im)	0.32 ± 0.08	1.11 ± 0.37	2.57 ± 0.99	2.18 ± 0.38	2.24 ± 0.25	1.91 ± 0.40	1.20 ± 0.22
Tumor(sr)	0.67 ± 0.16	1.52 ± 0.75	5.30 ± 1.62	4.66 ± 1.66	3.50 ± 1.81	2.88 ± 1.41	3.16 ± 1.15
Tumor(ih)	0.78 ± 0.37	2.87 ± 0.69	4.89 ± 1.60	4.41 ± 1.15	3.82 ± 1.17	3.12 ± 0.78	2.42 ± 0.70
Testicle	0.11 ± 0.01	0.15 ± 0.03	0.37 ± 0.05	0.32 ± 0.07	0.19 ± 0.02	0.15 ± 0.03	0.09 ± 0.01
Liver	1.34 ± 0.20	1.12 ± 0.34	0.76 ± 0.28	0.49 ± 0.10	0.46 ± 0.17	0.26 ± 0.05	0.17 ± 0.04
Kidney	1.38 ± 0.04	1.25 ± 0.15	0.98 ± 0.09	0.75 ± 0.11	0.59 ± 0.04	0.49 ± 0.04	0.40 ± 0.05
Lung	1.11 ± 0.15	0.81 ± 0.19	0.76 ± 0.08	0.69 ± 0.18	0.40 ± 0.02	0.33 ± 0.04	0.23 ± 0.03
Bone	0.08 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.00	0.02 ± 0.01	0.01 ± 0.01
Bone marrow	0.98 ± 0.26	0.77 ± 0.06	0.67 ± 0.05	0.48 ± 0.14	0.32 ± 0.05	0.19 ± 0.04	0.13 ± 0.04
Blood	4.20 ± 0.27	3.18 ± 0.24	2.45 ± 0.15	1.93 ± 0.47	1.21 ± 0.08	0.75 ± 0.08	0.51 ± 0.21

* n = 3

6.4 Activity distribution and tumor targeting capacity of radioimmunoconjugates with and without biotinylation

The antibody chiBR96 was labeled with ^{125}I, ^{111}In or ^{188}Re under identical conditions with about one or less than one chelate/conjugate coupling to each antibody. After having investigated a series of biotinylation levels, it was found that 2-3 biotin molecules coupled to each chiBR96 resulting in 80-95% avidin-gel binding capacity without significantly interfering with the immunoreactivity of the immunoconjugates. When more biotin molecules are coupled to each chiBR96 there is a high risk of interference with the antigen binding capacity of the antibody, while less than 2 biotin molecule coupling decreased the avidin-gel binding capacity. Table 5 shows the tumor targeting capacity of iodine-, indium- and rhenium-radiolabeled chiBR96 with and without biotinylation both *in vitro* and *in vivo*. A

similar activity distribution in normal tissues has also been obtained for biotinylated and non-biotinylated chiBR96 radiolabeled with one of these three radionuclides (^{125}I, ^{111}In or ^{188}Re) (Papers II, III, V).

A similar biodistribution and tumor targeting capacity between biotinylated and non-biotinylated radiolabeled-chiBR96, also demonstrated that the endogenous biotin, biotin-binding protein or biotin receptor has little interfering effect on the biotin-coupled immunoconjugates.

Table 5: Comparison of *in vitro* and *in vivo* tumor targeting capacity between biotinylated and non-biotinylated chiBR96 radiolabeled with ^{125}I (with NSTBB conjugate), ^{111}In (with chelate SCN-Bz-CHX-A-DTPA) or ^{188}Re (n = 4) (mean ± SD).

	^{125}I-chiBR96		^{111}In-chiBR96		^{188}Re-chiBR96	
	biotinylated	non-biotinylated	biotinylated	non-biotinylated	biotinylated	non-biotinylated
In vitro cell binding (%)	52 ± 5	55 ± 4	63 ± 6	54 ± 7	47 ± 6	52 ± 4
In vivo tumor uptake*	2.5 ± 0.7	2.9 ± 0.2	5.6 ± 0.7	6.3 ± 1.2	3.5 ± 1.1	4.5 ± 1.1

* at 24 h pi for tumor (sr) or (ih), (%/g)

6.5 Radioactivity uptake and retention in tumor tissue of biotinylated chiBR96 labeled with ^{125}I, ^{111}In or ^{188}Re

Different from non-internalizing antibodies, radiolabeled chiBR96 will be intensively catabolyzed in endosomes and lysosomes after binding to the epitope of the tumor cells. Activity uptake and retention in the tumor tissue of internalizing MAbs, such as chiBR96, not only depend on the stability of the radiolabeled immunoconjugates but also on the redistribution of the radioactive metabolites after having been catabolized in the tumor cells. A comparison was made using the same animal model, between ^{125}I-, ^{111}In- and ^{188}Re-labeled and biotinylated chiBR96, with optimized labeling methods, e.g. ^{125}I-chiBR96 labeled with NSTBB, ^{111}In-chiBR96 labeled with SCN-CHX-Bz-A-DTPA and ^{188}Re-chiBR96 with TFP MAG_2-GABA. The tumor uptake of the biotinylated chiBR96 labeled with ^{125}I, ^{111}In and ^{188}Re is shown in Figure 6. The investigation time schedule was identical for ^{125}I- and ^{111}In-labeled chiBR96. The activity retention in tumor tissue (%) increased up to 48 h post-injection for ^{125}I and then decreased (Paper IV, Fig. 2). In contrast, the tumor activity retention was increased for the whole of the investigation period of 8 days for ^{111}In-chiBR96 (Paper II, Fig. 3). The

specific activity of biotinylated ^{111}In-chiBR96 was 3 and 8 times higher than that of the biotinylated ^{125}I-chiBR96 at 8 days post-injection in (im) tumor and (sr/ih) tumor, respectively. A 3-day investigation period was used for biotinylated ^{188}Re-chiBR96 because of the short physical half-life, which was probably somewhat too short to determine the catabolism phase of the immunoconjugates.

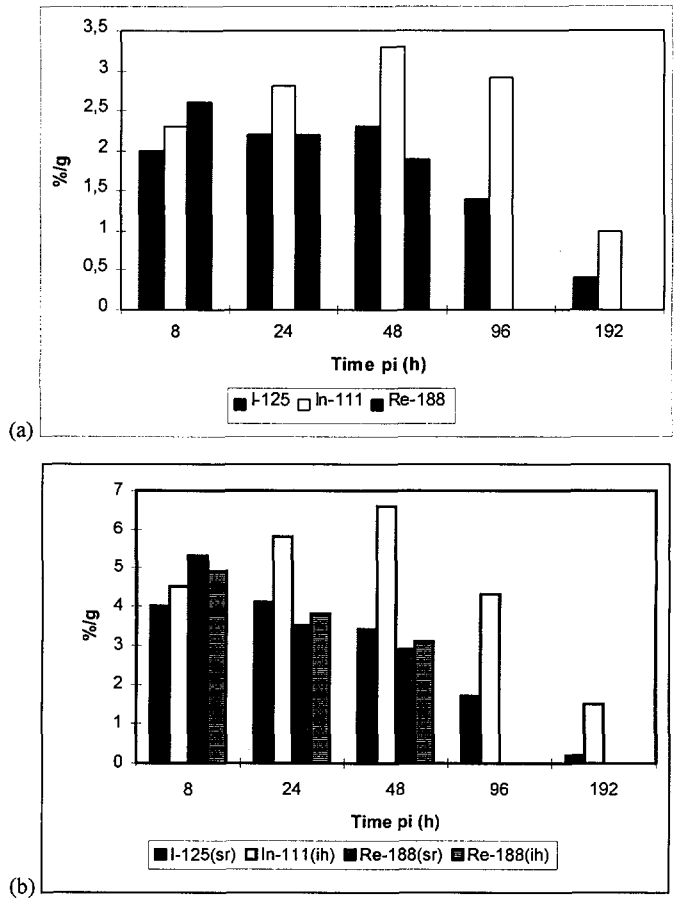


Figure 6: Tumor activity uptake, (a) tumor(im); (b) tumor(sr) or tumor(ih), of biotinylated chiBR96 labeled with ^{125}I, ^{111}In or ^{188}Re (') in colon carcinoma isografted rats at different times post-injection (n=6).

' The activity uptake of biotinylated ^{188}Re-chiBR96 was measured at 6, 24 and 48h pi.

6.6 Normal tissue distribution of biotinylated chiBR96 labeled with ^{125}I, ^{111}In or ^{188}Re

The activity distributions in normal tissues of interest for ^{125}I- and ^{111}In-labeled and biotinylated chiBR96, with labeling methods employing NSTBB and SCN-Bz-CHX-A-

DTPA, respectively, are listed and compared in Table 6. The biodistribution in normal tissues of interest were similar up to 24 h post-injection. The activity uptakes of ^{111}In-chiBR96 were higher than those of ^{125}I-chiBR96 in most normal tissues after 48 h post-injection. The activity accumulation of ^{111}In in liver and spleen was more than 10 times higher than ^{125}I at 8 days post-injection.

Table 6: Comparison of activity distributions in some normal tissues of interest for ^{125}I-, ^{111}In- and ^{188}Re-labeled and biotinylated chiBR96 in colon carcinoma grafted BN rats at different post-injection times (%/g) (n=5) (mean ± 1SD).

		8 h	24 h	48 h	96 h	192 h
Blood	^{125}I	2.6 ± 0.2	1.5 ± 0.3	0.9 ± 0.1	0.4 ± 0.1	0.05 ± 0.06
	^{111}In	2.1 ± 0.2	1.4 ± 0.2	1.1 ± 0.1	0.6 ± 0.2	0.13 ± 0.08
	^{188}Re		1.2 ± 0.1	0.8 ± 0.1		
Testis	^{125}I	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.01 ± 0.01
	^{111}In	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.34 ± 0.07
	^{188}Re		0.2 ± 0.0	0.2 ± 0.0		
Kidney	^{125}I	0.9 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.03 ± 0.02
	^{111}In	0.8 ± 0.2	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.39 ± 0.06
	^{188}Re		0.6 ± 0.0	0.5 ± 0.1		
Liver	^{125}I	1.1 ± 0.1	0.7 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	0.06 ± 0.02
	^{111}In	1.4 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.0	1.70 ± 0.39
	^{188}Re		0.5 ± 0.2	0.3 ± 0.1		
Spleen	^{125}I	0.6 ± 0.1	0.6 ± 0.2	0.3 ± 0.2	0.2 ± 0.0	0.13 ± 0.02
	^{111}In	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	1.18 ± 0.61
	^{188}Re		0.3 ± 0.0	0.2 ± 0.0		
Pancreas	^{125}I	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	0.04 ± 0.04
	^{111}In	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.05 ± 0.01
	^{188}Re		0.2 ± 0.0	0.2 ± 0.0		
Lung	^{125}I	1.0 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.03 ± 0.03
	^{111}In	1.0 ± 0.2	0.6 ± 0.0	0.6 ± 0.1	0.3 ± 0.1	0.17 ± 0.04
	^{188}Re		0.4 ± 0.2	0.3 ± 0.0		
Bone marrow	^{125}I	0.7 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.03 ± 0.02
	^{111}In	0.6 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.12 ± 0.08
	^{188}Re		0.3 ± 0.1	0.2 ± 0.0		

The difference in the activity distributions of ^{125}I- and ^{111}In-labeled MAbs is most possibly due to the fact that radioactive metabolites have different clearance rates from whole body, the ^{111}In-metabolites having a longer biological half-life (Jönsson BA et al, 1992). The good stability of the ^{111}In-labeled immunoconjugates resulted in a similar activity distribution between ^{125}I- and ^{111}In-labeled chiBR96 up to 24 h post-injection. A higher liver uptake would

already have occurred at this point in time if the ^{111}In-immunoconjugate had been unstable (Fig. 5, ^{111}In-chiBR96 using chelate CDTAMA or BADTPA). At 8 days after antibody administration, the activity distribution corresponded to the biodistribution of the radioactive metabolites rather than the immunoconjugates as most of the radiolabeled immunoconjugates have probably been catabolyzed at this time.

Owing to the short physical half-life of ^{188}Re and the 3-day investigation period of biotinylated ^{188}Re-labeled chiBR96, the normal tissue activity distribution at only 24 and 48 h post-injection are listed in Table 6. It was impossible (little activity of ^{188}Re could be measured after 3 days) to compare the ^{188}Re data with those of ^{125}I- and ^{111}In-labeled chiBR96 after 3 days post-injection. However, the activity distribution of the biotinylated ^{188}Re-chiBR96 was very similar to that of ^{125}I-chiBR96 during 72 h post-injection, except for 5-10% of the whole body activity accumulated in the colon, which was probably due to the catabolism of the ^{188}Re-labeled immunoconjugates and part of the ^{188}Re-chelate being excreted in feces (Beaumier et al, 1991).

6.7 Kinetics of biotinylated chiBR96 labeled with ^{125}I, ^{111}In or ^{188}Re

Table 7: Comparison of activity clearance from blood and whole body for ^{125}I-, ^{111}In- and ^{188}Re-labeled and biotinylated chiBR96 in colon carcinoma isografted BN rats.

	Blood		Whole body		
	$T_{1/2\alpha}$	$T_{1/2\beta}$	mono-phase $T_{1/2}$	$T_{1/2\alpha}$	$T_{1/2\beta}$
^{125}I-chiBR96	5 h (50%)*	38 h (50%)	-	6 h (12%)	73 h (88%)
^{111}In-chiBR96	4 h (47%)	61 h (53%)	161 h (100%)	-	-
^{188}Re-chiBR96	7 h (42%)	32 h (58%)	64 h (100%)	-	-

*Corresponding percentage of the activity in blood and whole body, respectively.

The rates of activity clearance from the blood and whole body of ^{125}I-, ^{111}In- and ^{188}Re-labeled and biotinylated chiBR96 are listed in Table 7. The activities of iodine-, indium- or rhenium-radiolabeled and biotinylated immunoconjugates were eliminated from blood with a bi-phase elimination curve. A distribution half-life ($T_{1/2\alpha}$) of 4-7 hours corresponding to about half of the blood activity was observed for each of the three radiolabeled and biotinylated chiBR96, while the $T_{1/2\beta}$ of ^{111}In-chiBR96 was significantly longer than that of ^{125}I- and ^{188}Re-labeled immunoconjugates. In contrast to about 12% of the activity of the biotinylated ^{125}I-chiBR96 being excreted from the whole body with a short half-life of 6 hours, the activity of ^{111}In- and

^{188}Re-labeled immunoconjugates were eliminated from the whole body with a mono-phase elimination curve. The ^{125}I and ^{188}Re was excreted from the body more than twice as fast as ^{111}In.

6.8 T/N ratio enhancement of ECIA

TABLE 8: Enhancement of radioactivity ratios of tumor(ih)-to-normal tissue using ECIA (n=5) and avidin chase (n=4) compared with controls (n=4) (mean ± SD).

	8 h pi			14 h pi	
	ECIA	**Avidin chase**	**Controls**	**ECIA**	**Controls**
Time of ECIA or "chase"	6 h	6 h		12 h	
Muscle	178.5 ± 44.4*	106.8 ± 71.2	115.1 ± 30.0	78.7 ± 24.5	84.7 ± 5.2
Testis	15.8 ± 2.9*	13.3 ± 2.5	11.3 ± 3.3	17.0 ± 1.2	14.4 ± 3.3
Liver	9.8 ± 1.7**	2.7 ± 0.3*	5.6 ± 2.7	23.7 ± 6.2 *	9.2 ± 1.2
Spleen	25.4 ± 7.1**	10.3 ± 1.2	10.6 ± 3.3	40.3 ± 8.8*	15.4 ± 4.0
Kidney	10.8 ± 3.2*	4.4 ± 0.6	5.7 ± 1.3	12.8 ± 3.2*	6.1 ± 1.5
Pancreas	38.3 ± 13.3*	15.3 ± 4.5	19.2 ± 3.7	32.5 ± 5.5*	16.7 ± 1.3
Stomach	35.7 ± 12.1	16.0 ± 1.5	21.4 ± 7.3	31.1 ± 7.1	23.7 ± 3.7
Small intestine	20.4 ± 3.8	9.8 ± 3.7	13.3 ± 8.1	28.4 ± 6.9*	12.2 ± 1.1
Colon	33.3 ± 25.4*	7.8 ± 5.1	14.1 ± 8.7	21.4 ± 6.8	10.3 ± 2.3
Heart	22.4 ± 7.3*	10.3 ± 3.5	10.6 ± 2.7	25.0 ± 5.0**	10.2 ± 2.0
Lung	13.1 ± 5.5*	6.2 ± 1.2	5.9 ± 1.6	14.4 ± 4.2*	5.7 ± 1.2
Bone marrow	18.2 ± 4.2**	7.2 ± 0.5	8.1 ± 2.6	35.6 ± 8.3**	9.9 ± 1.4
Blood	11.3 ± 5.1**	1.8 ± 0.4	2.0 ± 0.5	11.8 ± 3.4**	2.3 ± 0.4

* $P < 0.05$; **$0.01 < P < 0.05$.

The *in vivo* tumor targeting capacity of the biotinylated and radiolabeled MAb was not significantly changed compared with that of the non-biotinylated radiolabeled MAb (Papers II-III), which is a prerequisite for ECIA based on biotin-avidin concept as well as for avidin chase. Based on the activity biodistribution and the kinetics of biotinylated ^{188}Re-chiBR96 in colon carcinoma isografted BN rats, ECIA was started at 6 or 12 hours post-injection in order to remove as much blood activity as possible while retaining a high tumor uptake. More than 80% of the blood activity was removed by ECIA in both 6 and 12 hours post-injection time groups. Forty to seventy percent of the activity in blood-rich normal tissues, such as liver, lung, kidney and bone marrow was also removed by ECIA due to both the blood content in the tissues and the re-distribution of radiolabeled immunoconjugates in the extracellular fluid during the ECIA procedure (Paper VI). The tumor uptake was not significantly reduced by the ECIA. The T/N activity ratio was increased by a factor of 6 for the blood, and was significantly increased for blood-rich normal tissues, e.g. liver, kidney, lung and bone marrow (Table 8). Similar results were obtained by our group for biotinylated ^{125}I-chiBR96 in the

same animal tumor model (Garkavij et al, in press). The tumor(ih) and tumor(sr) were only visualized after a reduction in the background using ECIA procedure (Figure 7).

(a) (b)

Figure 7: Whole-body imaging of a rat, carrying colon carcinoma isografts intramuscularly in the thigh (im), beneath the subrenal capsule (sr) and intrahepatically (ih) administrated with 100 mg biotinylated ^{188}Re-chiBR96. (a) before ECIA at 6 h pi, (b) after ECIA at 8 h pi (arrows indicate tumors).

6.9 ECIA vs. avidin chase with respect to T/N ratio enhancement

Paper VI presents the first direct comparison of the ECIA and avidin chase approaches in the same animal tumor model with respect to T/N activity ratio enhancement. Biotinylated ^{188}Re-chiBR96 was employed, and both of the procedures were started at 6 hours post-injection. Different results were obtained due to the different mechanisms of these two methods. With the direct removal of the blood radioimmunoconjugates by ECIA, the T/N activity ratios were significantly increased for most normal tissues. No enhancement of T/N activity ratios was observed following the avidin chase, as the blood activity was soon restored after a significant reduction during the first 30 min (Paper VI, Fig. 2). More biotin molecules would have to be coupled to each antibody to increase the removal efficacy of blood activity by avidin chase, but this would also increase the risk of damaging the antigen binding capacity of chiBR96 (Paper VI). The mechanism behind the removal of blood activity by avidin chase is the formation of avidin-biotin-immunoconjugates which significantly increases the activity accumulation in the liver.

7. DISCUSSION

The use of radioimmunoconjugates combined with the biotin-avidin system, e.g. two- or three-step strategies, has recently been under investigation in an effort to enhance the T/N radioactivity ratio, which would lead to improve tumor visualization in RID and improve therapeutic effects in RIT (Goodwin et al, 1996). The limitations of pre-targeting approaches are described in the Introduction. Our general strategy of ECIA, also based on the biotin-avidin system, requires: A) that the MAb is both biotinylated and radiolabeled, and B) an avidin-gel affinity column. In this thesis, methods have been described for the internalizing MAb chiBR96 combining biotinylation and radiolabeling with each of three potentially therapeutic radionuclides, iodine, indium and rhenium, as well as their related different radiolabeling methods of iodine and indium being compared and optimized.

Biotinylated ^{111}In-chiBR96 with the aid of chelate SNC-CHX-Bz-A-DTPA, demonstrated an improved *in vivo* stability and tumor targeting capacity compared with labeling with the chelates CDTAMA or BADTPA. Conjugate NSTBB has been successfully used for iodination of chiBR96 combined with biotinylation, in contrast to combination with electrophilic labeling methods which seem to be particularly difficult to use. Moreover, a higher tumor uptake was obtained for biotinylated ^{125}I-chiBR96 labeled with the NSTBB conjugate, compared with the non-biotinylated ^{131}I-chiBR96 labeled with Iodogen method. Biotinylated ^{188}Re-chiBR96, radiolabeled with the aid of the chelate TFP MAG_2-GABA, showed a biodistribution similar to that of iodinated chiBR96. Recently, NSTBB and other similar conjugates have been used for MAb radiolabeling with other halogens, such as ^{211}At (Zalutsky et al, 1994;) and ^{76}Br (Wilbur and Hylarides, 1991). Clinical trials with ^{90}Y-labeled MAbs cc49 and m195 using the same chelate, SNC-CHX-Bz-A-DTPA, have been carried out and are still in progress (Scheinberg et al, 1996). Hence, the methods established in the present investigation give us a vast choice of different radionuclides for antibody labeling for use in RIT, as well as for potential use of the ECIA approach based on the biotin-avidin concept to enhance the T/N ratio, and consequently improve the therapeutic effect of RIT.

Regarding internalizing MAbs, the redistribution of the radioactive metabolites of the radioimmunoconjugates, after having been catabolized in the target cells, will significantly interfere with the tumor activity uptake and retention (Fritzberg and Beaumier, 1992). Activity localization with radiolabeled internalizing MAb has been determined *in vitro* with cultured target tumor cells (Mattes et al, 1994; Shih et al, 1994a), and the characteristics of

internalizing MAbs (which are different from those of non-internalizing MAbs) affecting therapeutic efficacy in RIT are under consideration (Stein et al, 1995; Shih et al, 1994b; Reist et al, 1995).

Once the antibody is internalized and catabolized in the lysosomes, the radioactive metabolites become a key factor for the activity retention in the target cells. It is known that some of the radioactive metabolites, such as iodotyrosine and iodobenzoic acid, are rapidly exit the lysosomes. Some of the radioactive metabolites, as free indium/yttrium released from the chelate under the low pH in lysosome, are trapped within the lysosomes essentially due to their inability to cross the lysosome membrane. In the present investigation, we compared the biotinylated and simultaneously radiolabeled internalizing MAb, chiBR96, with each of the three most commonly used radionuclides (iodine, indium and rhenium) in a colon carcinoma isografted rat model. The tumor activity uptake and retention were significantly improved through the use of cellularly retained radionuclides, i.e. ^{111}In-labeled MAb compared with iodine- or rhenium-labeled MAb. Although the iodination and rhenium labeling methods described in the present studies, have been optimized with an improved *in vivo* stability of the radioimmunoconjugates, the activity retention in the tumor tissue, especially ^{125}I-chiBR96 was significantly reduced 2 days post-injection. This may be due to the extensive catabolism of the MAb after endocytosis in the endosome and lysosome, and fast excretion of the ^{125}I-conjugate (Paper III).

In order to increase the retention of activity in tumor tissue after endocytosis of internalized MAb, residualizing iodine radiolabels, e.g. using cellobiose-tyramine (Ali et al, 1990; Ali et al, 1988) and dilactitol-tyramine (Strobel et al, 1985; Stein et al, 1995) have recently been investigated. The tumor activity retention has been increased with residualizing iodine labels, but the radioactivity accumulation in the normal tissues was also increased, and there was no significant improvement in the T/N activity ratio (Ali et al, 1990). Also, another limitation is the low radiochemical yield; only 3-6% radioiodination efficiency was obtained (Stein et al, 1995). A new conjugate, N-succinimidyl 5-(tri-n-butylstannyl)-3-pyridinecarboxylate (SPC) (Fig. 2), which has previously been used in the comparison of different conjugates for *in vivo* stabilization of iodinated MAb by Zalutsky's group (Garg et al, 1993), has been recently reported in internalizing MAb iodination experiments resulted in an enhanced activity retention in tumor tissues (Zalutsky, 1996).

By selecting radionuclides which are cellularly retained, as $^{111}In/^{90}Y$ as well as residualizing iodine radiolabels, the activity retention (lysosomally trapped) is increased not only in tumor cells, but also in normal tissues involved in antibody catabolism such as liver, spleen and possibly other tissues, as well as in any antigen expressing normal cells (Shih et al, 1994). The removal of circulating radioimmunoconjugates before they have been catabolized can decrease the background. In the present studies, the biotinylated and simultaneously radiolabeled chiBR96 with one of three radionuclides showed similar activity distributions within the first 24 hours after antibody administration. This results suggested that the high activity accumulation in the normal tissues was due to the catabolism of the immunoconjugates and slow systemic clearance of the radioactive metabolites of ^{111}In-chiBR96, and that the activity of biotinylated ^{111}In-MAb in the circulation could be removed by the ECIA approach, just like the removal of biotinylated ^{125}I-chiBR96 and biotinylated ^{188}Re-chiBR96. After a period of tumor targeting, the removal of circulating $^{111}In/^{90}Y$ and residualizing radioiodine labeled immunoconjugates, may enhance the T/N ratio with improved tumor activity retention.

Different from non-internalizing MAbs, pre-targeting approaches, which require that the MAb remains on the tumor cell surface for a while for amplifying targeting of the radioactivity, are irrespective of internalizing MAbs. In contrast, the present investigation demonstrated that more than 80% of the blood activity of biotinylated ^{188}Re-chiBR96 is removed by ECIA, and that the background in the blood-rich normal tissues is greatly reduced resulting in an enhanced T/N ratio. Moreover, the fast internalization of the immunoconjugates after binding to the epitope minimizes the redistribution of the radioimmunoconjugates within target tissues after ECIA or avidin chase, if the radioactive metabolites can be trapped in the target cell lysosomes.

A direct comparison of ECIA and avidin chase, both of them relying on MAb biotinylation and simultaneous radiolabeling, demonstrated that avidin chase was not as effective as ECIA under the present investigated conditions. A higher level of biotinylation is necessary to ensure the formation of the avidin-biotin-immunoconjugate complex (Marshall et al, 1994). Repeated avidin chases, or injection of streptavidin, which has a longer biological half-life, instead of avidin, may increase the efficacy (Kobayashi et al, 1995). The problems of antigenicity of the avidin/streptavidin (Schreiber et al, 1995) and the accumulation of complexes with a high activity in liver and spleen, will still hamper the use of avidin chase in

RIT. Avidin chase might be an effective diagnostic procedure to increase the visualization of tumor lesions located outside the reticuloendothelial system by removing blood activity (Paganelli et al, 1990).

8. CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis has led to several interesting findings.

1. A method has been developed for simultaneous biotinylation and radiolabeling of MAb chiBR96.
   a. ChiBR96 biotinylation and indium labeling using the chelate SCN-Bz-CHX-A-DTPA shows improved *in vivo* stability and tumor targeting capacity of the radioimmunoconjugates compared with two other chelates investigated in this study.
   b. Conjugate NSTBB has been successfully used for iodination of chiBR96 combined with biotinylation, in contrast to the combination with electrophilic labeling methods, which seem particularly difficulty to use. The tumor uptake and T/N activity ratio are improved using biotinylated ^{125}I-chiBR96 with NSTBB conjugate compared with that of ^{131}I-chiBR96 iodinated with iodogen method.
   c. ChiBR96 simultaneously biotinylated and ^{188}Re labeled with chelate TFP MAG_2-GABA demonstrates a similar activity distribution to that of corresponding iodinated MAb.
2. Through the direct removal of circulating radioimmunoconjugates, ECIA significantly enhances the T/N activity ratio for biotinylated ^{188}Re-chiBR96, and exhibits some advantages over avidin chase in the same animal tumor model.
3. For internalizing MAb chiBR96 the tumor activity uptake and retention have been significantly increased by selecting a cellularly retained radionuclide ^{111}In, instead of iodine or rhenium.
4. The similar normal tissue biodistributions of chiBR96 biotinylated and radiolabeled with ^{111}In, ^{125}I and ^{188}Re within 48 h post-injection, demonstrated that the ECIA approach has a considerable ability to remove the background of ^{111}In-labeled immunoconjugates, and promptly improve and maintain T/N activity ratios.

Future perspectives

On the basis of the methodology established in this study, many of the proposed therapeutically useful radionuclides, such as halogens, rhenium and some of the radiometals, could be used for MAbs radiolabeling and simultaneous biotinylation. Absorbed dose calculations, based on the biodistribution and kinetics of corresponding radionuclide-labeled

immunoconjugates, can be used as a guide in choosing the most suitable radionuclide in order to obtain the maximal improvement in therapeutic effect using ECIA.

Tumor radioactivity uptake and retention will be increased using residualizing iodine labeling method, and radiometals, e.g. ^{111}In-^{114m}In, especially when internalizing MAbs are used. ECIA will be a very efficient method of removing the high-activity background providing an enhanced and maintained T/N activity ratio. In order to increase the radioactivity uptake and retention in the tumor tissue, MAb chiBR96 ^{90}Y labeling and simultaneous biotinylation, and potential use of ECIA with biotinylated ^{90}Y-chiBR96 should also be necessary.

Future investigations should concentrate on finding the potential improvement of tolerable administered radioactivity with ECIA for the maximal therapeutic effect enhancement.

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