Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin

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Due to their high affinity for biotin, avidin (Av) and streptavidin (SAv) are used to bridge pretargeted antibody molecules and radiolabeled biotin derivatives in vivo. **Methods:** We compared uptake of ^{125}I-labeled Av or SAv (~10–500 μg) in tumor and normal tissues 3 days after a biotinylated B72.3 monoclonal antibody (100 μg) injection in nude mice. The animals were killed 24 hr later and the biodistribution of ^{125}I was determined. **Results:** The percent injected dose per gram of tumor remained constant over the range of injected doses for Av while that for SAv varied. As larger amounts of Av/SAv were injected, the number of moles of each trapped within tumor increased, with the values for SAv being much higher. While the injection of larger doses of Av led to an increase in tumor-to-normal tissue ratios, that of SAv did not. **Conclusion:** SAv (2.5 mg/kg) is the preferred "second-step" reagent. At this dose, the number of receptors available for targeting by radiolabeled biotin derivatives is ~1.8 times the number of antigen-binding sites accessible for targeting by radiolabeled antibody. Additional targeted-signal amplification should be possible by the successive and repeated administration of such polymeric reagents, each exhibiting high affinity to and forming a specific binding pair with the last-targeted molecule.

Key Words: avidin/streptavidin; biotin; monoclonal antibody; signal augmentation; tumor targeting

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Monoclonal antibodies (MAb) have generally been considered particularly attractive as selective carriers of diagnostic/therapeutic agents due to their unique in vitro specificity and high affinity for their antigen. Their application in humans for both diagnosis (labeled with isotopes of iodine, ^{99m}Tc and ^{111}In) and therapy (labeled with the beta emitters ^{131}I, ^{186}Re, ^{90}Y and ^{67}Cu, and the alpha emitters ^{211}At and ^{212}Bi) continues to be the focus of attention in many research laboratories.

In all such studies, the basic assumption continues to be that radiolabeled MAbs have a role in radioimmunodiagnosis (RID) and radioimmunotherapy (RIT). The low percent injected dose per gram (%ID/g) of target tissue and the nonuniform distribution of MAbs within the targeted tumor, however, have dampened the early enthusiasm of a decade ago and have led some investigators to question the future of MAbs in the clinic (*1*). Despite various opinions on the subject (*2–7*), it is clear that unless alternative approaches addressing the most obvious shortcoming of MAbs (i.e., low %ID/g) are developed, MAbs are unlikely to be used routinely in medicine.

Several approaches have already been described in which an antibody that is not internalized by the targeted cell is injected prior to the administration of a small radiolabeled molecule that has a strong affinity to the antibody (*8–27*). The most extensively studied approach utilizes the high avidity of avidin (Av) or streptavidin (SAv) for biotin, a 244-Da vitamin found in low concentration in blood and tissues. Because the noncovalently bound Av/SAv-biotin complex has an extremely low dissociation constant (k_d) of about 10^{-15} *M* (Table 1), investigators have used these two molecules as binding pairs to bridge molecules that have no affinities for each other and to target various radionuclides. For example, biotinylated antibodies have been administered to animals several days prior to the injection of radiolabeled Av or SAv (*8,10,11,15,16,24,26*). Alternatively, radiolabeled biotin derivatives have been synthesized and used as carriers of radioactivity to pretargeted Av/SAv-MAb conjugates or following the consecutive injection of biotinylated antibody and Av/SAv (*8,12,18,19,21,22,24,25*).

It is clear that the successful utilization of a radiolabeled molecule (e.g., radiolabeled MAb) in RID or RIT is mainly dependent on the ability to achieve both a high percent injected radioactive dose per gram of targeted tissue and favorable target-to-normal tissue ratios (T/NT). In all pretargeting approaches, it is also important to determine the number of moles of the pretargeted molecule as this will certainly impact on the probability of the radiolabeled molecule complexing with the last pretargeted molecule. For example, in a MAb-biotin$_n$●●●Av/SAv●●●radiolabeled biotin approach, the number of Av/SAv molecules that bind to the pretargeted biotinylated MAb at the tumor should correlate with the %ID/g of radiolabeled biotin molecules trapped at the targeted tissue (Fig. 1). In addition, since several biotin molecules can be conjugated per MAb molecule without measurable losses in immunoreactivity (*28,29*) and there are four biotin complexation sites per Av/SAv molecule, such approaches should lead to the augmentation of the original targeted signal.

Recently, we have been examining the concept of target-signal augmentation (TSA) in several systems in which a pair of polymeric molecules with no affinity for each other are bridged using a polymeric molecule that has high affinity for both of these molecules (*32*). In one of these systems, a noninternalized biotinylated antibody is injected first and the targeted signal is then amplified by the repeated and successive administration of Av/SAv and polybiotin derivatives (e.g., polybiotinylated human serum albumin) prior to the injection of a radiolabeled species (e.g., biotin, Av/SAv). In this paper, we have examined and compared the abilities of Av and SAv to amplify the targeted signal after biotinylated MAb administration.

MATERIALS AND METHODS

B72.3 Production and Assay

The murine anti-human mammary cancer MAb B72.3 (IgG_1) was used. This MAb is not internalized by the LS174T cells in vitro (*33,34*) and has been used extensively to target LS174T tumors. As such, this MAb is most appropriate for the proposed in vivo pretargeting approach.

B72.3-producing hybridoma cells, HB8108, were obtained from the American Type Culture Collection (Rockville, MD). Cells were

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TABLE 1
Properties of Avidin and Streptavidin*

	Avidin	Streptavidin
Source	Egg white	Bacteria
Biotin receptors	4	
k_d (Biotin)	10^{-15} M	
Dissociation from biotin ($T_{1/2}$)	200 days	
Molecular weight	~64,000	~71,000
Carbohydrate	yes	no
Isoelectric point	10	5–6
Nonspecific adsorption	high	low
$T_{1/2}$ in blood	minutes	hours

*From Refs. 28–31.

grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Long Island, NY) supplemented per 500 ml with 10% fetal bovine serum, sodium pyruvate (1 ml of 10 m*M* stock), L-glutamine (1 ml of 20 m*M* stock), penicillin-streptomycin (5 ml of 5000 units/ml stock) and fungizone (0.5 ml of 250 μg/ml stock). Cells were injected intraperitoneally in male BALB/c mice. B72.3 was purified from ascites by affinity chromatography over Protein A/G (Pierce, Rockford, IL) and a concentration of ~3 mg/ml was stored at −20°C in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% sodium azide.

Immunoreactivity was determined by an indirect radioimmunoassay which was based on the method described by Carney et al. (*35*). Ninety-six well microtiter plates (Costar, Cambridge, MA) were coated with 100-μl aliquots (100 mg/ml) of Type I bovine submaxillary mucin (Sigma Chemical Company, St. Louis, MO) in 10 m*M* Tris-HCl, pH 7.4, and incubated overnight at room temperature (RT). They were then washed in distilled water and incubated for 2 hr with PBS containing 0.1% bovine serum albumin (BSA). Plates were washed three times in PBS and serial dilutions (1:2) of B72.3 in 1% BSA were added. Following a 1 hr, 37°C incubation, the plates were washed five times with PBS to remove unbound antibody, ^{125}I-labeled rabbit antimouse IgG (~100,000 cpm/100 μl) (NEN Research Products, Du Pont Company, Boston, MA) diluted in PBS was added, and the plates were reincubated for 1 hr at 37°C. Finally, the plates were washed five times and the bound ^{125}I activity was determined in a gamma counter.

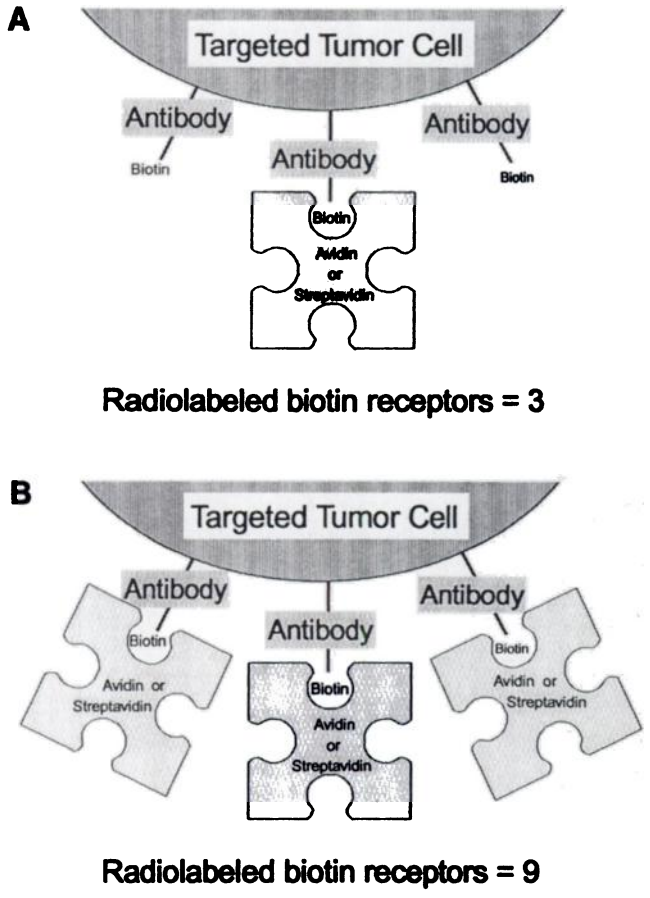

FIGURE 1. Schematic of targeted cell●●●biotinylated-antibody●●●avidin/streptavidin interaction when nonsaturating (A) and saturating (B) amounts of Av/SAv are administered.

Radiolabeling of Proteins

B72.3 (600 μg/600 μl PBS) was radiolabeled with Na^{125}I (300 μCi) using Iodogen (20 μg) as the oxidizing agent. Following a 5-min incubation at RT, the reaction mixture was loaded onto a Sephadex G-25 column prewashed with PBS containing 1% BSA. The protein was eluted with PBS and the fractions containing ^{125}I-B72.3 were pooled and concentrated. Radiolabeling yields >85% were usually obtained.

Avidin and streptavidin, obtained from Sigma Chemical Company, were iodinated using ^{125}I-Bolton-Hunter Reagent (NEN Research Products, Du Pont Company). In brief, 2 mg of Av/SAv in 200 μl PBS, pH 8.0, were added to an ice-cold test tube coated with ~300 μCi ^{125}I-Bolton Hunter Reagent. The reaction was stopped after 15 min by the addition of 0.4 ml of 0.2 *M* glycine in PBS, pH 8.0. Unreacted ^{125}I-Bolton-Hunter Reagent was removed by gel filtration on a Sephadex G-25 column.

Biotinylation of B72.3

B72.3 was concentrated 10-fold by centrifugation through a Centricon-30 concentrator (Amicon, Beverly, MA), then resuspended in 50 m*M* NaH_2CO_3, pH 8.5, to a final concentration of 2 mg/ml. An aqueous solution of NHS-LC-biotin (Sigma Chemical Company) in 10-fold molar excess was added and the mixture incubated on ice for 2 hr. To remove unreacted biotin, the solution was centrifuged in a Centricon-30 concentrator (2 × 15 min, 5000 × *g*) and the retentate assayed for both immunoreactivity (see above for method) and the number of moles of biotin bound per mole of antibody. The latter was determined by titrating biotin against avidin as described by Der-Balian (*36*). Briefly, biotinylated B72.3 (33 pmole) was added to 120 pmole avidin and the fluorescence of the sample was measured using a Perkin Elmer L50B Luminescence Spectrometer, with an excitation wavelength of 300 nm and an emission wavelength of 347 nm. Readings were then made following the addition of 5-μl aliquots of 5 μ*M* biotin.

Pharmacokinetic Studies

LS174T cells obtained from the American Type Culture Collection were grown in Dulbecco's Modified Eagle Medium supplemented per 500 ml with 10% heat-inactivated fetal bovine serum, nonessential amino acids (5 ml of 10 m*M* stock), L-glutamine (5 ml of 200 m*M* stock) and penicillin-streptomycin (5 ml of 5000 units/ml). Athymic mice (nu/nu, BALB/c background), 5–6-wk-old, were obtained from Harlan Sprague Dawley, Incorporated (Indianapolis, IN). Mice were injected subcutaneously with one million LS174T cells. Subcutaneous tumors were palpable 8 days after cell injection.

To establish the biodistribution of B72.3, mice were injected with ~5 μCi ^{125}I-B72.3 (10 μg) intravenously via a lateral tail vein. The mice (five/group) were killed over a 5-day period, and the tumor and the following organs and tissues were removed, weighed, and counted in the gamma counter: liver, spleen, kidneys, small intestine, large intestine, stomach, bladder, lung, heart, skin

and muscle. The radioactive contents of a small volume of blood and urine were also assayed.

To determine the effect of avidin and streptavidin dose on the uptake of these reagents, mice (five/group) were intravenously injected with 100 μg biotinylated B72.3 via the lateral tail vein. Seventy-two hours later, 10–20 μg (~5 μCi) of either ^{125}I-avidin or ^{125}I-streptavidin, to which 0–500 μg of the corresponding nonradiolabeled species had been added, were injected by the same route. The animals were killed 24 hr later and the tumor, normal tissues and organs were weighed and counted in the gamma counter.

Calculations

To assess the advantages/disadvantages of an indirect approach (e.g., biotinylated antibody●●●Av/SAv●●●radiolabeled biotin) over the direct approach (radiolabeled MAb), it is essential to determine and compare the number/moles of receptors for each within the targeted and nontargeted tissues (biotin receptors in Av/SAv and antigen-binding sites, respectively) at the highest T/NT ratios (i.e., where the target-to-normal tissue amplification is maximal).

To calculate the number of antigen-binding sites occupied by the biotinylated MAbs, the percentage of the injected radioactive dose per gram of tissue, organ and tumor is multiplied by the number of moles of MAb (IgG) injected per mouse (100 μg/150,000 or 6.67 $\times 10^{-10}$ mole). For example, since the %ID/g of tumor at 72 hr for the radiolabeled MAb B72.3 is 28.5, the number of moles of antigen targeted by 100 μg biotinylated antibody per gram of tumor is 1.9×10^{-10} (it is assumed that one MAb molecule binds per antigen).

To calculate the number of biotin receptors within the various tissues, organs and tumor, the number of moles of Av/SAv were determined (percent injected radioactive Av/SAv dose/g multiplied by μg/64,000 or μg/71,000, respectively) and the values multiplied by three (it is assumed that one of the four biotin receptors per Av/SAv is utilized to complex these molecules to one biotin molecule on the pretargeted biotinylated MAb thus leaving three receptors available for the next targeted molecule). For example, since 0.4% of the injected 3.9×10^{-9} moles of Av (249 μg/64,000 $\times 10^{-6}$) was bound to the tumor, 1.5×10^{-11} moles Av are present per gram of tumor. This is equivalent to 4.7×10^{-11} moles of biotin receptors/g tumor for this injected dose.

To calculate the signal amplification factor (i.e., the ratio of receptors that can be occupied by the radiolabeled molecule to be injected next), the number of moles of biotin receptors calculated is divided by those of antigen. In the above example, the original antigenic signal has therefore been amplified 0.24 times.

This approach necessitates that the T/NT ratio and the degree of signal amplification are simultaneously used to determine the most favorable dose of Av or SAv that should be administered. For Av, this occurs when 249 μg are injected (Fig. 4).

RESULTS

Window of Opportunity Determination

A biodistribution study of ^{125}I-B72.3 was performed in nude mice bearing ~200–300 mg of tumor to determine the optimum time for injection of the "second-step" reagent (i.e., Av/SAv). The results (Fig. 2) demonstrate that after ^{125}I-MAb administration the radioactive content of the blood and all normal tissues and organs declined with time, whereas the activity within the tumor increased over the first 24-hr period and stayed at high levels (~28 %ID/g) over the next 72 hr. The data also indicate that the most favorable tumor-to-normal tissue ratios were obtained 72 hr after MAb injection. In all subsequent studies, this time period was selected as the "window of i.e., the time to administer Av/SAv.

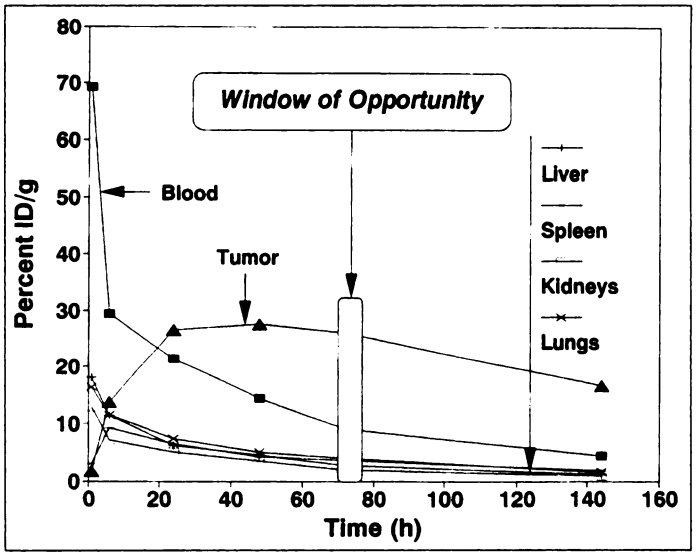

FIGURE 2. Iodine-125 uptake (%ID/g) in LS174T subcutaneous tumors, various organs, tissues and blood after administration of ^{125}I-B72.3. Note that highest T/NT ratios are obtained at 72 hr.

Pharmacokinetics of Avidin Versus Streptavidin in Blood

The blood clearance of Av and SAv was determined in normal mice. As previously reported (*11,31,37*), the results show that ^{125}I-avidin cleared more rapidly than ^{125}I-streptavidin from the circulation (both curves were biphasic). At 24 hr, the SAv levels in the blood (%ID/g) were approximately 30–80 times greater than those of Av.

Biodistribution of ^{125}I-Avidin after B72.3-Biotin Administration

Tumor-bearing animals were injected with increasing concentrations of avidin (18 μg ^{125}I-Av + 0–462 μg cold Av) 72 hr after the administration of 100 μg biotinylated B72.3 (MAb-B_4), and the mice were killed 24 hr later. The results indicate that the %ID/g of tumors remained constant (0.36 ± 0.05) over the range of injected doses (Fig. 3). On the other hand, the %ID/g of normal tissues (with the exception of the kidneys) generally decreased when increasing doses of Av (18–249 μg) were administered. Consequently, excluding the kidneys, the tumor-to-normal tissue ratios increased as a function of injected Av dose (Fig. 4).

As mentioned above, one of our goals was to determine whether an increase in the injected Av dose would increase the number of biotin receptors within the targeted tumor without a concomitant rise in normal tissues. The results (Fig. 5) demonstrate that the number of moles of Av in both tumor and normal tissues increased with higher doses of administered Av, albeit to different degrees, and that at the highest injected dose (480 μg), the number of biotin receptor sites within the tumor was approximately half the number of antibody molecules that targeted the tumor (Table 2).

Biodistribution of ^{125}I-Streptavidin after B72.3-Biotin Administration

In these studies, 72 hr after the administration of 100 μg of biotinylated B72.3 (MAb-B_2), tumor-bearing animals were injected with increasing concentrations of streptavidin (12 μg ^{125}I-SAv + 0–485 μg cold SAv). The mice were killed 24 hr later, and the radioactivity within the tumor and normal tissues was determined. The results indicate that unlike Av, where the %ID/g of tumors remained constant over the range of Av

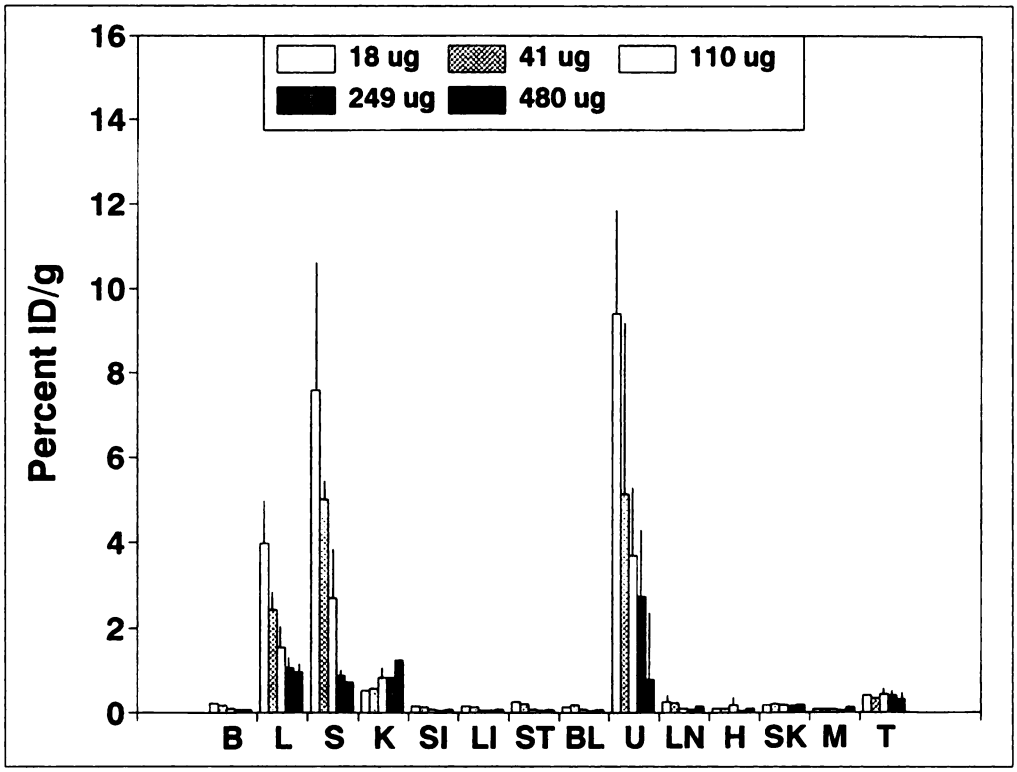

FIGURE 3. Two-step biotinylated antibody●●●Av approach. Iodine-125 uptake (%ID/g) in LS174T tumors, various organs, tissues, urine and blood after injection of ^{125}I-Av + increasing concentrations of nonradiolabeled Av. Mice were pretreated with biotinylated B72.3 (MAb-B_4) 72 hr earlier. B = blood; L = liver; S = spleen; K = kidneys; SI = small intestine; LI = large intestine; ST = stomach; BL = bladder; U = urine; LN = lung; H = heart; SK = skin; M = muscle; T = tumor. Bar = s.d.

opportunity'' for the B72.3 MAb-LS174T animal tumor model, concentrations administered, the %ID/g of SAv increased first (47-μg injected dose) to a high of approximately 17% but then decreased when higher doses were administered (Fig. 6). These SAv %ID/g within the tumors are 13–53 times greater than those obtained with Av. On the other hand, the %ID/g of SAv in normal tissues (with the exception of liver) remained constant. Consequently, the highest T/NT ratios were observed at the 47-μg SAv dose (~2.5 mg/kg body weight) decreasing thereafter as a function of increasing SAv dose (Fig. 7). When these percentages are converted to the number of moles within target and normal tissues, the results demonstrate that the amount of SAv increased with higher doses of administered SAv (Fig. 8). It is important to note that the absolute values of SAv are approximately 10–20 times greater than those obtained for Av (compare the y-axis scales in Figs. 5 and 8). In addition, the number of biotin receptor sites within the tumor at the SAv dose that gave the highest T/NT ratios (47 μg) was approximately 1.8 times the number of antigen targets to which radiolabeled antibody molecules could bind (Table 2).

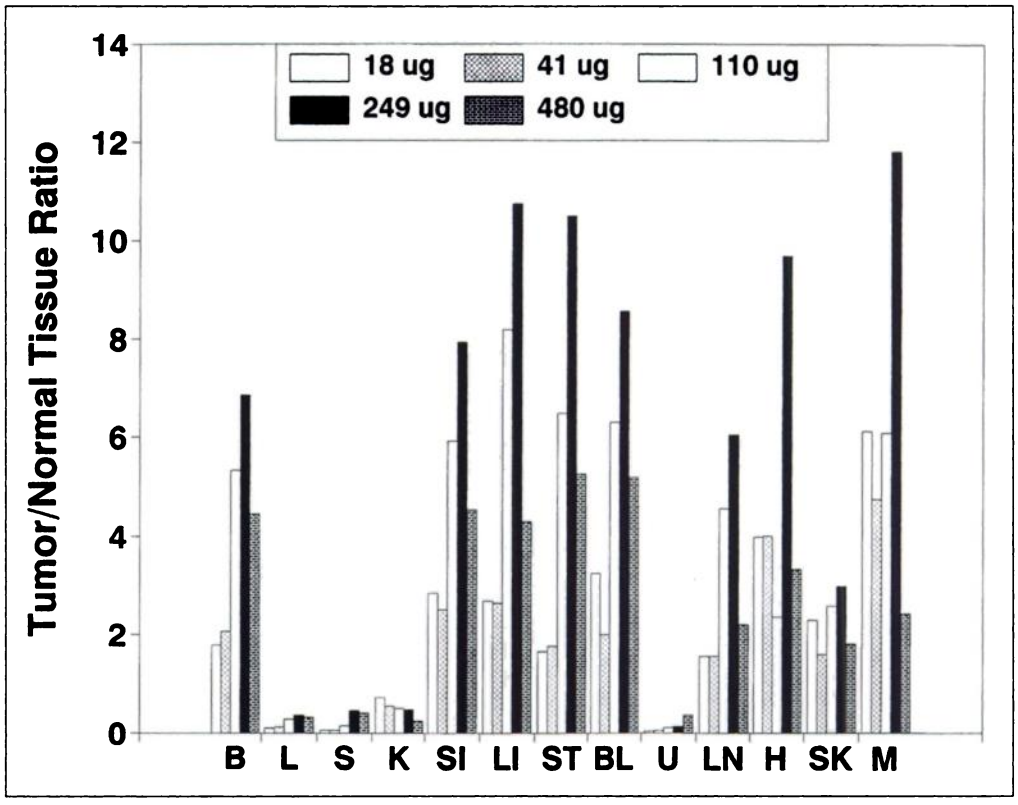

FIGURE 4. Two-step biotinylated antibody●●●Av approach. Tumor-to-normal tissue ratios calculated from data in Figure 3 (see legend of Fig. 3 for abbreviations).

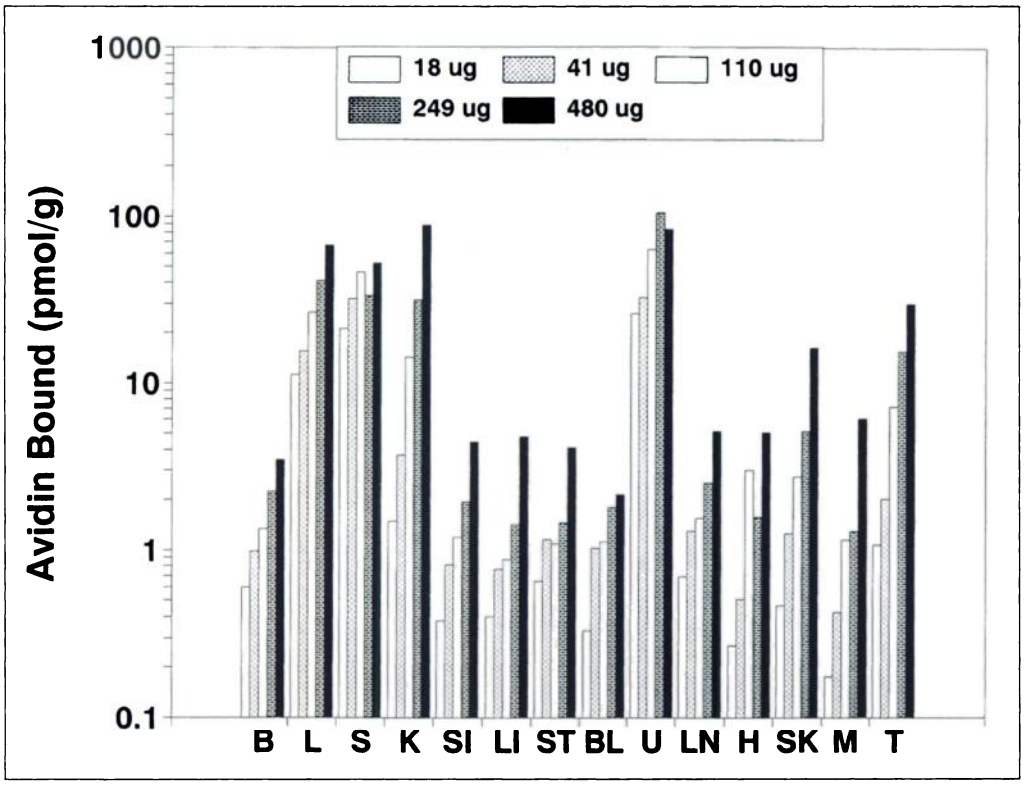

FIGURE 5. Two-step biotinylated antibody●●●Av approach. Avidin concentrations (pmole/g) within LS174T tumors, various organs, tissues, urine, and blood are calculated from data in Figure 3 (see legend of Fig. 3 for abbreviations).

DISCUSSION

The ability of radiolabeled MAbs to bind tumor-associated antigens has been utilized to target various radionuclides to tumors for both diagnosis and therapy. For clinical applications, one of the major limitations in such targeting continues to be the low percent injected dose per gram of targeted tumor. Several approaches have addressed this critical shortcoming. For example, it has been shown that interferon enhances the expression of certain tumor-specific antigens (*38, and references therein*) and leads to an increase in the binding of radioimmunoconjugates (*39,40*). Tumor targeting of radiolabeled MAbs has also been shown to be enhanced by the prior injection of a covalent complex of the MAb and vasoactive molecules (*41,42*).

Strategy for Target Amplification

Recently, we have been examining several approaches that aim to amplify the targeted signal prior to the administration of the diagnostic or therapeutic agent. The strategy is based on the concept of injecting repeatedly and in succession polymeric molecules that have high affinities for each other (*32*). As the molecules bind to each other, the originally targeted signal is continuously amplified (Fig. 9). Finally, a radiolabeled molecule that has a high affinity to the pretargeted molecules is administered. In one of these systems, which is the subject of this paper, several biotin molecules are conjugated to an antibody that is not internalized by its target (e.g., tumor cells). When such a biotinylated antibody is administered to tumor-bearing animals and a certain percentage binds to its intended target, each antigen-binding site to which an antibody molecule complexes will be amplified by the number of biotin molecules that are conjugated to the antibody. The targeted signal will then be further augmented by the repeated and successive administration of avidin or streptavidin and a polybiotinylated molecule, for example polybiotinylated human serum albumin or biotin dentrimers (Fig. 9). Finally, radiolabeled biotin (when the last administered molecule is Av/SAv) or radiolabeled Av/SAv (if polybiotin was injected last) is administered. Because of the tetrameric nature of Av and SAv (i.e., each molecule has four biotin-binding sites) and the polymeric nature of the biotinylated molecule, signal amplification should increase in an exponential fashion as the cycling of these binding partners continues (Table 3; Fig. 9). It is important to note that the degree to which the targeted signal is amplified during the cycling process will depend on (1) the extent to which all the sites within the last pretargeted molecule have been occupied (Fig. 1), and (2) the ability of Av and SAv to bind four biotin molecules each. While the validity of this statement has been repeatedly demonstrated for nonconjugated biotin, Green (*43*) has demonstrated that the four binding sites within Av are not evenly distributed with two sites being close together and that each pair of sites binds only one biotinylated protein. As such, an avidin molecule is capable of acting as a bridging pair between two biotinylated proteins but not, as it is commonly stated, as an amplifying factor. Although this will have an effect on the extent to which the signal is amplified per cycle (Table 3), it does not alter the percent theoretical expectation values shown in Figure 10. Finally, it is also important to recall that the

TABLE 2
Target-Signal Amplification (TSA) in LS174T Tumors

	Avidin				
Injected dose (μg)	18	41	110	249	480
Specific activity (Ci/mole)	1.84E5	8.10E4	3.02E4	1.22E4	6.92E3
TSA	0.017	0.032	0.11	0.24*	0.47
	Streptavidin				
Injected dose (μg)	12	47	97	247	497
Specific activity (Ci/mole)	2.96E4	7.55E3	3.66E3	1.44E3	7.14E2
TSA	0.29	1.77*	1.98	2.89	4.45

*Dose at which the most favorable tumor-to-normal tissue ratios are obtained (see Figs. 4 and 7).

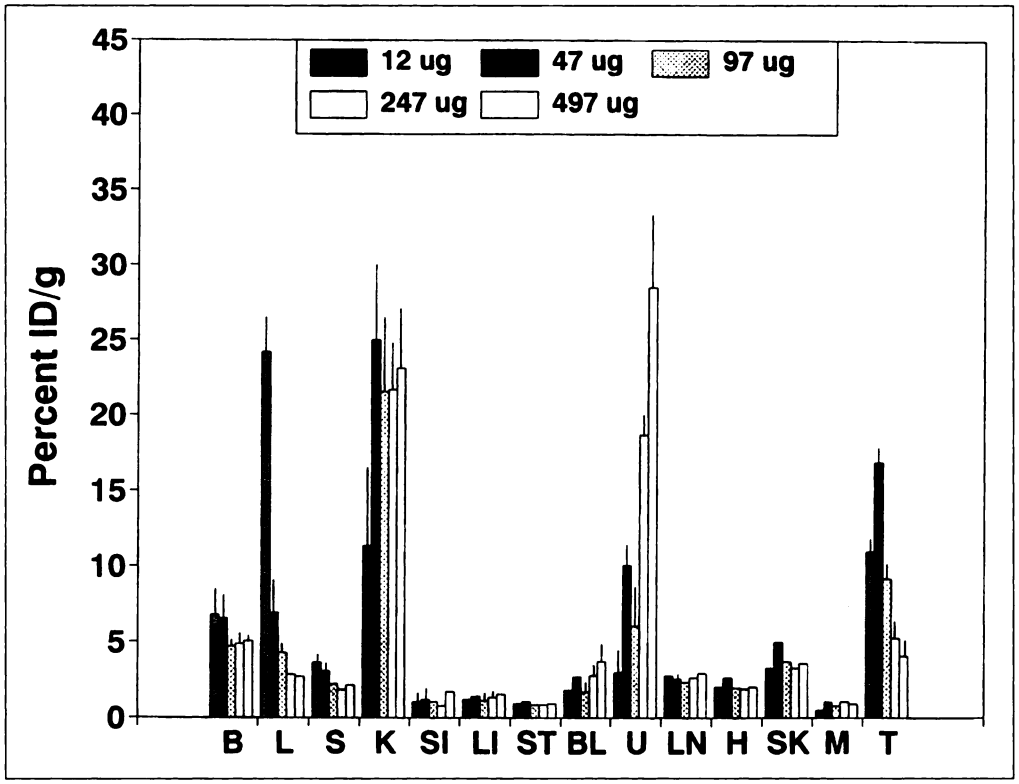

FIGURE 6. Two-step biotinylated antibody●●●SAv approach. Iodine-125 uptake (%ID/g) in LS174T tumors, various organs, tissues, urine and blood after administration of ^{125}I-SAv + increasing concentrations of nonradiolabeled SAv. Mice were preinjected with biotinylated B72.3 (MAb-B_2) 72 hr earlier (see legend of Fig. 3 for abbreviations). Bar = s.d.

success of such an approach will strongly depend on the specificity of the biotinylated antibody, high T/NT ratios, and the absence/minimal internalization of the antibody, the antibody●●●Av/SAv complex, and all the complexes formed by the repeat injections of the polymeric reagents used prior to the administration of the radiolabeled moiety.

Target Signal Amplification: Avidin versus Streptavidin

Although avidin and streptavidin recognize the same binding sites on biotin and have approximately the same affinity and molecular weight (Table 1), Av is glycosylated and SAv is not. This difference has been reported by some to contribute to the latter molecule's lower in vitro nonspecific adsorption to various surfaces and molecules (*28,30*) and slower in vivo clearance (*11,31,37*). Consequently, the probability for interaction between each of these two molecules and a preinjected biotinylated moiety in vivo is expected to differ. Such factors as well as the concentration of the pretargeted polybiotinylated antibody within the target and in normal tissues will in turn affect the %ID/g of tumor and normal tissues, the number of moles at the target, and the T/NT ratios. Naturally, the viability

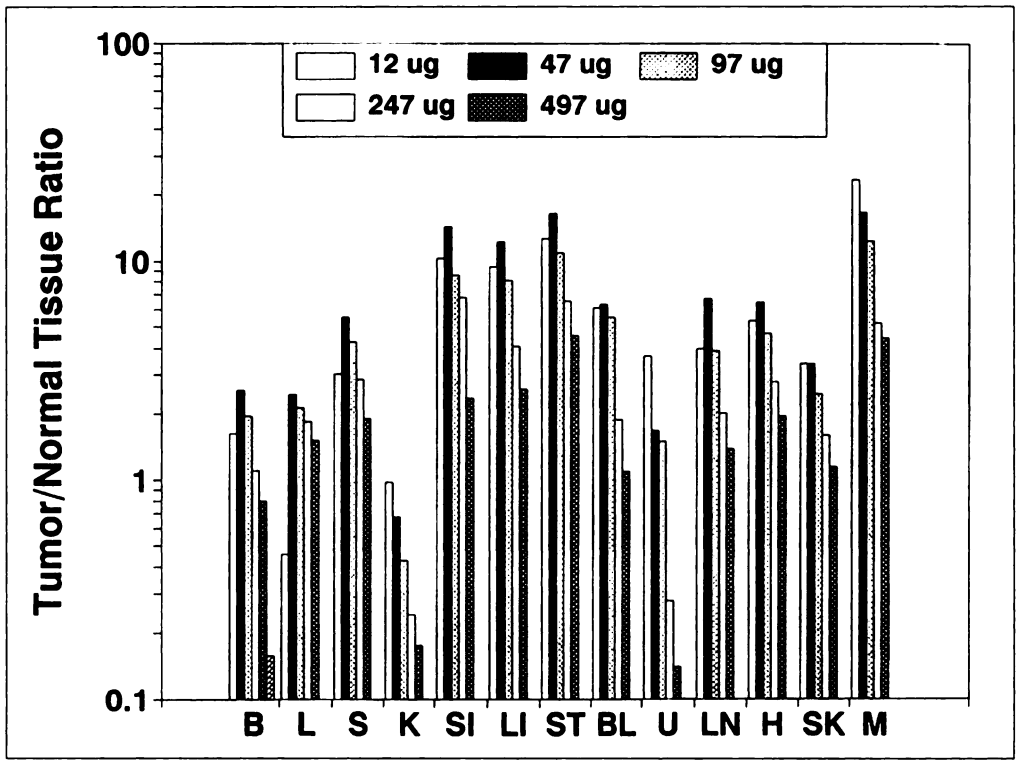

FIGURE 7. Two-step biotinylated antibody●●●SAv approach. Tumor-to-normal tissue ratios calculated from data in Figure 6 (see legend of Fig. 3 for abbreviations).

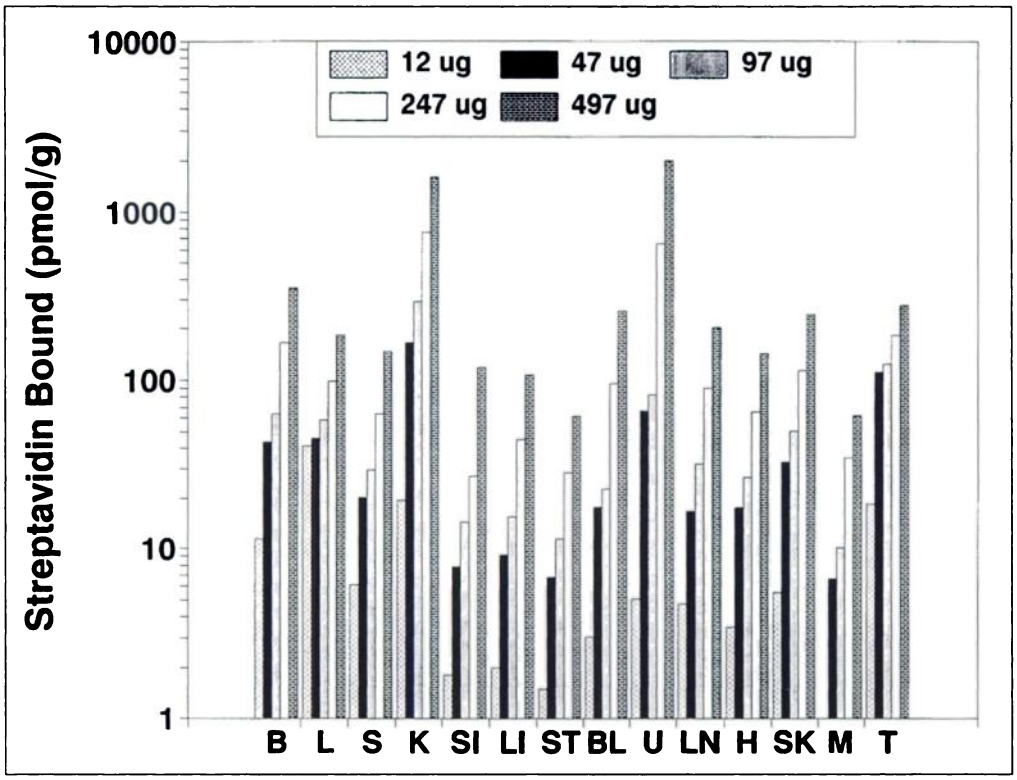

FIGURE 8. Two-step biotinylated antibody●●●SAv approach. Streptavidin concentrations (pmole/g) within LS174T tumors, various organs, tissues, urine and blood are calculated from data in Figure 6 (see legend of Fig. 3 for abbreviations).

of any amplification approach necessitates that the signal be continuously augmented to a greater degree at the targeted tumor than in normal tissues (i.e., it is essential that the residence time of the targeted molecules [e.g., polybiotinylated antibody] and/or the complexes formed between the polymeric molecules [e.g., polybiotinylated antibody and Av or SAv] is longer within the targeted tumor than within normal tissues thus leading to T/NT ratios that are >1). In this paper, we have examined and compared the abilities of Av and SAv to amplify the targeted signal after biotinylated MAb administration and have assessed the effect of dose (μg Av/SAv) on the uptake of the corresponding radiolabeled species in tumor and normal tissues. Obviously, such information is relevant for establishing the optimal conditions when a third-step probe is to be used in such systems.

The results corroborate our expectations and clearly demonstrate (Table 2) target-signal augmentation (TSA) following the administration of both Av and SAv, albeit to different degrees (~0.02 to ~0.5 and ~0.3 to ~4.5 times, respectively). While T/NT ratios, however, were highest when ~12.5 mg Av/kg body weight were injected (Fig. 4, Table 2), the TSA values of <1 indicate the unsuitability of Av for such an approach. On the other hand, at the highest T/NT ratios for SAv (~2.5 mg SAv/kg body weight), the target signal was amplified by ~1.8 times (Fig. 7, Table 2) clearly demonstrating the appropriateness of SAv for the proposed amplification method. The reason for this difference is unclear; it may be related to the longer clearance rate of hours for SAv compared with that of several minutes for Av (*11,31,37*). Alternatively, this may be due to a differential processing of these two molecules by various tissues.

Advantages of Cycling

The proposed cycling approach has certain advantages over those in which antibodies are used as carriers of radioactivity or when a biotinylated antibody is pretargeted before the injection of a radiolabeled species (Av/SAv) or Av/SAv followed by radiolabeled biotin (*8,10–12,15,16,18,19,21,22,24–26*). First,

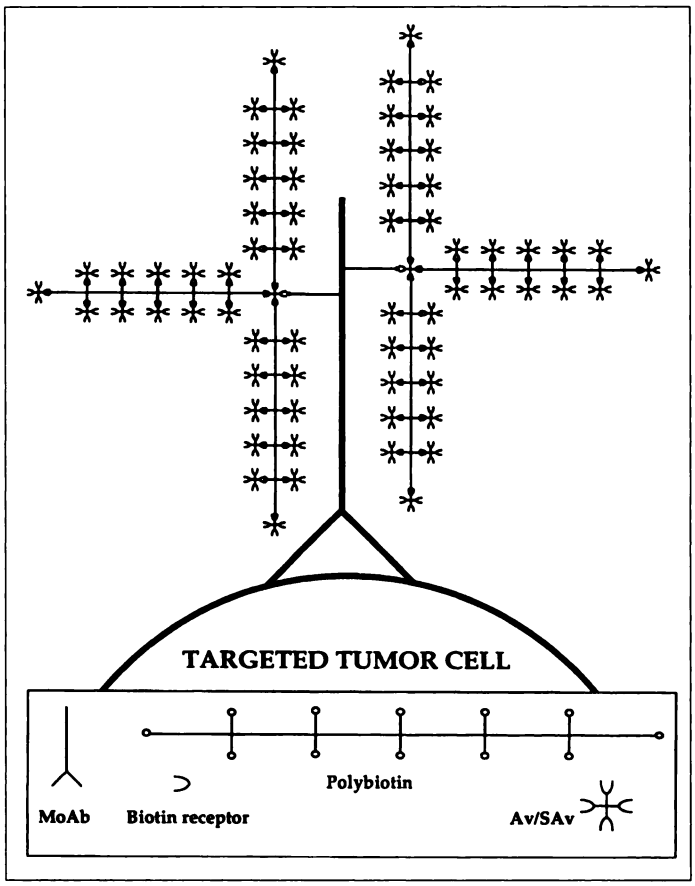

FIGURE 9. Schematic of target-signal augmentation (TSA). Biotinylated antibody (MAb-B_2) is pretargeted to tumor and Av/SA, polybiotin (B_{12}), and Av/SAv are injected in succession. Under these conditions, original antigenic signal to which antibody is bound has been amplified 198-fold. In other words, there are 198 sites to which radiolabeled biotin molecules can bind. In a three-step approach (MAb-B_2●●●Av/SAv●●●radiolabeled biotin), the original targeted signal would have been amplified six times (i.e., only six receptors for biotin at the target).

TABLE 3
Amplification of a Single Antigenic Signal: Theoretical Expectations

		Amplification factor	Signal amplified by *n = 2	Signal amplified by n = 4
Cycle 1	MAb-B_n	×2	2	4
	Av/SAv	× (4–1)	6	12
Cycle 2	M-$B^{\dagger}_{15}$	× (15–1)	84	168
	Av/SAv	× (4–1)	252	504
Cycle 3	M-B_{15}	× (15–1)	3,528	7,056
	Av/SAv	× (4–1)	10,584	21,168
	*M-B_{15}/*B‡			

*n = number of biotin molecules in MAb-biotin conjugates.
†Polybiotinylated molecule or polybiotin.
‡Radiolabeled polybiotinylated molecules (*M-B) or radiolabeled biotin (*B).

the radiolabeling of an antibody with various isotopes involves exposing it to different conditions, thereby causing distinctive changes in antibody structure and function. Such radiolabeled MAb preparations would be expected to have differing pharmacokinetics (*35,44*). In addition, the radiolabeled MAb may undergo radiolysis. The proposed approach, on the other hand, requires a single mild manipulation (i.e., biotinylation) of the MAb. Biotin has been conjugated to many antibodies, and this chemical modification has been shown to have a minimal effect on antibody immunoreactivity even when antibody to biotin ratios of 1:10 have been achieved (*29*). In our studies, the conjugation of up to four biotin molecules per B72.3 MAb molecule had no measurable effect on this antibody's immunoreactivity (i.e., similar to nonbiotinylated B72.3). Finally, since the MAb is not the radionuclide carrier, the problems associated with radiolysis of the MAb will not be encountered.

Second, in vitro studies have demonstrated that high antibody concentration improves the rate of antibody penetration and its overall uptake by the tumor, i.e., the targeting of many more antigen-binding sites (*45–48*). It is unrealistic, however, to expect to target most or all of the antigenic sites expressed by a tumor with a radiolabeled antibody in vivo, since this necessitates administering unacceptably large amounts of radioactivity or radiolabeling at low specific activity in which case most antibody molecules will not be radiolabeled and, therefore, even though the tumor will be targeted, most of the antibody molecules within it will be nonradioactive. In the proposed approach, the quantity of antibody administered and the duration of the injection can both be increased in an attempt to saturate many of the accessible and available antigen-binding sites, thereby maximizing the antibody concentration at the target (greater mole/g) and increasing its penetration and uniform distribution within the targeted tumor (*45,46*). MAb infusion should also lead to the specific targeting of a larger proportion of tumor cells as this increases the likelihood that the MAb will be available when cycling cancer cells whose antigen expression is cycle-dependent are being targeted.

Third, the proposed approach would be expected to augment the original signal. For example, under the hypothetical conditions described in Table 3 (Av and SAv, each containing four biotin binding sites; MAb-B_2 and M-B_{15}; i.e., two biotin molecules conjugated to a MAb and 15 biotin molecules conjugated to a molecule), a single original antigenic signal at a tumor cell would be amplified 252 times following MAb-B_2●●●Av/SAv●●●M-B_{15}●●●Av/SAv administration (two Av/SAv molecules can interact with each MAb-B_2 molecule = sixfold amplification; three M-B_{15} molecules can react with each Av/SAv molecule = 84-fold amplification; and 14 Av/SAv molecules can react with each M-B_{15} molecule = 252 biotin receptors). If it is assumed further that all the sites to be targeted (e.g., biotin receptor sites on Av/SAv and biotin molecules on M-B_{15}) can be reached, a simple calculation indicates that following two more cycles (M-B_{15}, Av/SAv; M-B_{15}, Av/SAv), the original signal could be amplified in theory 10,584 times prior to the injection of a polybiotinylated-radiolabeled molecule (*M-B_{15}). While it is apparent that many assumptions would have to be valid for these theoretical expectations to materialize (including target but not nontarget cell signal amplification), if only 1% of these expectations can be achieved, the signal should be amplified ~100-fold. In practice, a 10-fold increase would be sufficient to deliver a therapeutic dose of ~10,000 rad to a tumor (in general, the dose to human tumors following the injection of radiolabeled MAb and a targeting of 0.01 %ID/g is ~1,000 rad). In our studies (MAb-B_4●●●SAv), we achieved up to 4.5-fold target-signal amplification (Table 2), ~74% of the theoretical expectations (Fig. 10). The data indicated, however, that T/NT ratios were most favorable when ~50 μg of SAv were injected (Fig. 8). At this dose, T/NT ratios were >1 for all of the tissues (with the exception of kidneys) indicating that the degree to which the nontarget tissues are being amplified is less than that at the target. It is therefore clear that for SAv, maximal signal amplification (~1.8 times) within the targeted tumor (and therefore lesser signal amplification within nontumor tissues) occurs at this injected dose (~2.5 mg/kg) following the injection of MAb-B_2. Obviously, TSA can be doubled (~3.6 times) if the tumor-bearing animals are pre-injected with MAb-B_4.

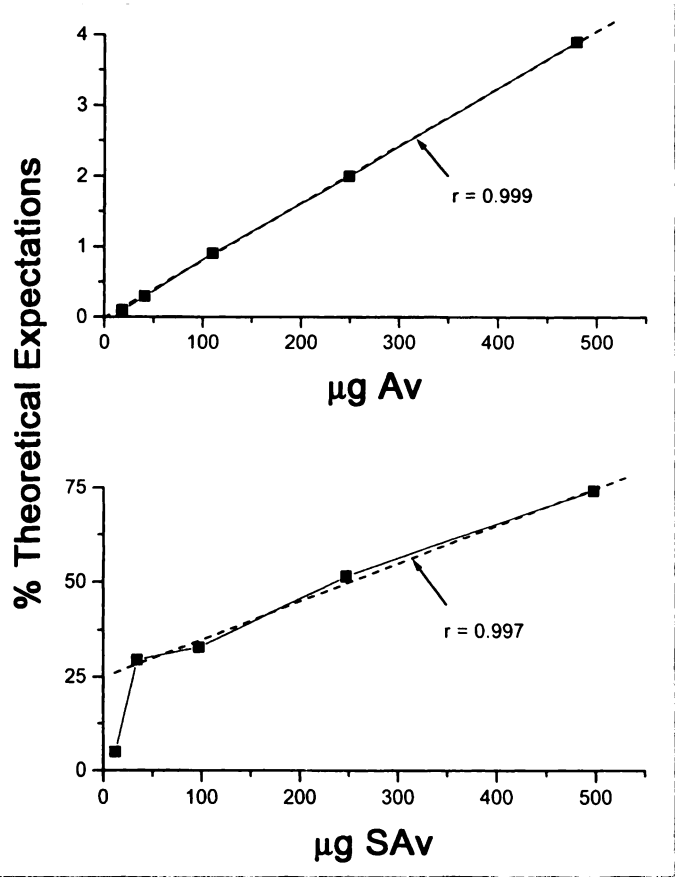

FIGURE 10. Plot of percent theoretical expectations as function of injected Av or SAv dose (tumor-bearing mice were injected with MAb-B_4●●●Av or MAb-B_2●●●SAv). Dashed lines represent linear fit of data points (for SAv, first point not included).

Fourth, the proposed approach allows flexibility in the choice of the molecule to be radiolabeled, the only restriction being that it must be amenable to both routine biotinylation and the chemistry inherent in the radiolabeling process. Since this molecule does not have any immunologic properties that must be protected, one need not be concerned about any functional losses following chemical manipulation. As such, it will also be possible to radiolabel the protein/peptide with any radionuclide (using established radiolabeling procedures) at extremely high specific activities. In addition, the size, charge, reactive groups, etc., of such molecules can be manipulated to produce a carrier moiety that has the required pharmacokinetic properties, e.g., low molecular weight to allow rapid targeting and bypass the high interstitial pressure within tumors (*49,50*).

Differences in Targeting Efficiencies of Avidin and Streptavidin

The data presented in this article also shed some light on an unexpected difference between Av and SAv. We wanted to address the question of how efficient were the two molecules at enhancing the targeted signal. Toward this end, the TSA values obtained (Table 2) were divided by the corresponding theoretical amplifications (Table 3) and the percent theoretical expectations (% TE) were calculated and plotted as a function of injected dose (Fig. 10). We assumed that the data would be fitted by a linear regression line, the extrapolation of which would go through the zero point. For avidin, this was the case ($r = 0.999$); however, the absolute % TE values were low (~0.01–~4). On the other hand, the data for SAv could not be fitted on a single straight line that could be extrapolated through zero: four of the five points fell on a straight line ($r = 0.997$) whose extrapolate had a Y intercept of ~25%. In addition, the SAv values were significantly higher (~5%–~75%) than those obtained with Av. The basis for this clear difference is not obvious. It may relate to the longer half-time of SAv in circulation (*11,31,37*) or to the nonspecific adsorption of Av (*28,43*). Further studies are necessary to address the reason(s) for these in vivo observations.

CONCLUSION

A targeted-signal augmentation approach has been described in which polymeric molecules (Av/SAv and polybiotin) with high affinities to each other are successively injected into tumor-bearing animals. The results indicate that prior to the injection of radiolabeled biotin derivatives, SAv (2.5 mg/kg) is the preferred "second-step" reagent for tumor localization studies. Since under these circumstances the experimentally measured TSA is ~1.8 times, the number of receptors available for targeting by radiolabeled biotin derivatives after the successive injection of MAb-B_4 and SAv is ~3.6 times the number of antigen-binding sites accessible for radiolabeled antibody targeting. These findings support the feasibility of target amplification in vivo and lead us to conclude that further signal augmentation will be possible by the successive repeated administration of polymeric reagents, each exhibiting high affinity to and forming a specific binding pair with the last-targeted molecule.

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Monoclonal Antibodies Labeled with Rhenium-186 Using the MAG3 Chelate: Relationship between the Number of Chelated Groups and Biodistribution Characteristics

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Our previous studies on the preparation of ^{186}Re-MAb conjugates for clinical radioimmunotherapy (RIT) were extended with the aim to derive conjugates which have a high Re:MAb molar ratio, are stable in vitro and in vivo, have favorable biodistribution characteristics and can be used together with ^{99m}Tc-MAb conjugates as a matched pair in combined radioimmunoscintigraphy/RIT studies. **Methods:** Rhenium and ^{99m}Tc-conjugates of intact MAb E48 were prepared according to our previously described multistep procedure using the MAG3 chelate and analyzed by protein mass spectrometry for the number of chelate molecules coupled to the MAb. For biodistribution analysis, tumor-free nude mice were simultaneously injected with ^{186}Re-, ^{99m}Tc/^{99}Tc- and/or ^{125}I-labeled E48 IgG and dissected 1–48 hr postinjection. **Results:** Rhenium-186-MAb conjugates with up to 20 Re-MAG3 groups per MAb molecule were prepared with an overall radiochemical yield of 40%–60%. The conjugates did not contain empty MAG3 groups and no aggregates were formed. Only conjugates with a ^{186}Re-MAG3:MAb molar ratio higher than 12 demonstrated slightly impaired immunoreactivity to a maximum of 15% decrease at the 20:1 molar ratio. Biodistribution experiments revealed that a proportion of the conjugate became rapidly eliminated from the blood for conjugates with a Re-MAG3:MAb molar ratio higher than 8. In this case, an increased uptake of activity was observed in the liver and intestines. The ^{99m}Tc/^{99}Tc-MAb conjugates showed a similar enhanced blood clearance when containing more than eight Tc-MAG3 groups, while dual labeling of MAbs revealed that the in vivo stability of the conjugated Re-MAG3 complex itself does not differ from the corresponding Tc-MAG3 complex. **Conclusion:** With the method described in this study, it is possible to prepare ^{186}Re-MAG3-MAb conjugates that fulfil all the aforementioned criteria for use in clinical RIT. Coupling of too many metal-MAG3 groups to MAbs results in rapid blood clearance. At the same metal-MAG3:MAb molar ratio, ^{99m}Tc/^{99}Tc-MAb conjugates show a similar pharmacokinetic behavior as ^{186}Re-MAb conjugates and can thus be used to predict the localization of ^{186}Re-labeled MAbs and make dosimetric predictions in individual patients.

Key Words: radioimmunotherapy; radioimmunoscintigraphy; rhenium-186-labeled monoclonal antibody; technetium-labeled monoclonal antibody; head and neck cancer

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The world wide incidence of squamous-cell carcinoma of the head and neck (HNSCC) is estimated at 500,000 cases a year (*1*). Patients with early stage disease (Stages I and II) are usually treated with surgery and radiotherapy and have a relatively good prognosis. Patients with Stages III and IV usually undergo combined surgery and radiotherapy but with limited success. In 50%–60% of these patients, locoregional recurrences occur after locoregional therapy, whereas 15%–25% develop distant metastases. Therefore, at least 30% of the HNSCC patients would benefit from the availability of an effective systemic adjuvant therapy. With respect to the application of (neo)adjuvant chemotherapy almost all studies, unfortunately, have failed to show any improvement in survival (*2,3*). One of the approaches to improve adjuvant therapy for patients is the use of monoclonal antibodies (MAbs) (*4,5*). Radiolabeled MAbs may be particularly suitable for the treatment of HNSCC due to the intrinsic radiosensitivity of this tumor type (*6*). To this end, we recently tested a panel of MAbs for targeting HNSCC in preclinical and clinical studies (*7–13*).

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