Development of New Multivalent-bispecific Agents for Pretargeting Tumor Localization and Therapy[1]

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Abstract

Purpose: **Two bispecific diabodies (BS1.5 and BS1.5H) and two bispecific trivalent proteins (BS6 and BS8) were produced and tested as potential agents for pretargeted delivery of radiolabeled bivalent haptens to tumors expressing carcinoembryonic antigen.**

Experimental Design: **Each of the four proteins was expressed in *Escherichia coli* and purified from the soluble fraction. BS1.5 and BS1.5H (a humanized version of BS1.5) were evaluated in the GW-39 human colonic tumor-nude mouse model using a di-HSG-1,4,7,10-tetra-azacyclododecane-*N*,*N*′,*N*″ *N*‴-tetraacetic acid peptide (IMP-241) radiolabeled with ^{111}In. The biodistribution and T/NT ratios were compared with those of hMN-14 × m679 (Fab′ × Fab′) prepared chemically.**

Results: **In animals, both BS1.5 and BS1.5H cleared more rapidly than hMN-14 × m679 and showed tumor to nontumor ratios far superior to those of hMN-14 × m679. For example, with BS1.5 injected 8 h before ^{111}In-IMP-241, the tumor uptake of ^{111}In was 10.3 ± 2.7 and 6.3 ± 2.2% ID/g at 3 and 24 h, respectively, with the tumor to blood ratios being 167 ± 35 at 3 h and 631 ± 231 at 24 h. In comparison, the tumor to blood ratios of ^{111}In observed for hMN-14 × m679 given 24 h earlier were 8 ± 2 at 3 h and 16 ± 3 at 24 h.**

Conclusions: **These results indicate that BS1.5 and BS1.5H are promising candidates for use in a variety of pretargeting applications, including tumor therapy with radionuclides and drugs. BS6 and BS8 may be even more attractive because of their potential to achieve higher levels of tumor uptake because of divalent carcinoembryonic antigen binding.**

Introduction

Since the first successful demonstration that radiolabeled antibodies could be used for cancer detection ~25 years ago (1), several antibody-based cancer-imaging agents have been commercialized. In addition, RAIT has reached an important development milestone with the recent approval of ibritumomab tiuxetan for the treatment of relapsed indolent NHL[3] (2). Solid tumors, however, have been less responsive to RAIT, principally because of their lower radiosensitivity, which requires a higher radiation dose to be delivered than what directly radiolabeled antibodies have been able to achieve (3). Despite this current limitation, targeted delivery of radionuclides for imaging and therapy of solid tumors continues to be a very active field of investigation (3).

To date, strategies for targeted delivery of radionuclides to tumor by antibody are basically divided into two approaches: one where the radionuclide is directly attached to the antibody, and the other where the nonradiolabeled antibody is first pretargeted to the tumor, with the radioactivity subsequently given at a later time (4). Because a directly radiolabeled antibody has slow clearance from the blood, a dose-limiting amount of radiation is most likely delivered to the red marrow before there is sufficient radioactivity accumulated in the more radioresistant solid tumors to cause significant antitumor effects. By giving the radioactivity that is associated with a small molecule, which can clear rapidly from the blood and body as a separate injection, there would be less radiation exposure to the red marrow, thereby decreasing toxicity and potentially improving the ability to localize greater amounts of radioactivity in the tumor. Although several pretargeting approaches have been reported, the most prominent methods involve the use of either a bsAb with a radiolabeled hapten or some variations of a procedure based on the interaction of streptavidin and biotin (4). In each of these procedures, the primary targeting agent (*e.g.,* an antibody-streptavidin, an antibody-biotin conjugate or a bsAb) is administered first and then the radioactive agent (the effector) is given at a later time. Ideally, the second step is implemented when the concentration of the primary targeting agent in the tumor is greater than in other tissues and when the blood concentration of the targeting agent is low enough so that the radioactive effector

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[3] The abbreviations used are: NHL, non-Hodgkin's lymphoma; AES, affinity enhancement system; bsAb, bispecific binding protein constructed chemically or recombinantly from two different monoclonal antibodies; CEA, carcinoembryonic antigen; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; HSG, histamine succinyl glycine; Ni-NTA, nitrilotriacetate complexed with nickel (II) ion; RAIT, radioimmunotherapy; RU, response unit; T/NT, tumor to nontumor.

molecule is not first entrapped by the targeting agent in the blood. For the bsAb pretargeting approach, hapten valency is a major determinant for tumor uptake and retention of an effector. Effectors that contain a bivalent hapten are markedly superior to those possessing a single hapten (5). This AES is believed to be attributable to the ability of the bivalent hapten to cross-link the pretargeted macromolecule at the tumor site, resulting in the formation of a more stable complex and, therefore, a longer tumor residence time (5). All streptavidin-biotin-pretargeting approaches have not used the divalent hapten, primarily because the affinity of radiolabeled biotin (effector) for streptavidin is already very high (4), and thus, affinity enhancement is not required to improve the stabilization of the effector binding to the pretargeted agent. Despite the higher binding affinity of streptavidin and biotin, the primary targeting agent in each of these pretargeting systems is anchored to the tumor by an antibody, and thus, the residence time of the radiolabeled effector in the tumor is essentially dependent on the avidity/affinity of the antibody for the tumor antigen.

Although pretargeting approaches were first used for improvements in T/NT ratios that enhanced imaging capabilities (4), interest in therapeutic applications for pretargeting advanced with the finding of Axworthy *et al.* (6) that by using their streptavidin-biotin-pretargeting approach, concentrations of a radiolabeled biotin localized in a tumor could rival those of a directly radiolabeled antibody. This pretargeting system has been optimized subsequently and shown to enhance therapeutic efficacy comparable with a directly radiolabeled antibody (7). Recently Press *et al.* (8) reported that a streptavidin-biotin-pretargeting procedure was more efficacious and less toxic in animal models with human lymphoma. Similarly, therapeutic improvements also were observed with a bsAb-pretargeting approach in animal models (9, 10). Clinical trials with the streptavidin-biotin approach showed some promising targeting results, and doses of ^{90}Y-biotin as high as 100 mCi/m^2 were tolerated (11–13). Gastrointestinal toxicity was a problem in this system, primarily because the antibody also had the capability of binding to the intestines (12). Clinical trials with a radioiodinated peptide using a bsAb-pretargeting approach have also been reported with some responses noted (14, 15). However, because of human antimouse antibody responses to the bsAb used in these early trials, this group is now evaluating a new bsAb that was prepared by coupling a humanized anti-CEA antibody (hMN-14) with a murine anti-DTPA antibody (m734) and appears to reduce the incidence of antiantibody responses (16, 17).

Our group has also begun evaluating pretargeting as an option for RAIT, opting to examine methods that use bsAb. The primary incentive for selecting this approach over that of streptavidin-biotin is that much of our efforts over the past years have been to prepare humanized versions of antibodies to reduce the immunogenicity of their murine counterparts (18–21). Thus, our decision was guided by our concern that streptavidin or avidin, however integrated in humanized antibody, would likely have considerable immunogenicity. Indeed, Weiden and Breitz (22) recently reported that 6 of 10 patients with NHL given a streptavidin-rituximab (chimeric anti-CD20) conjugate developed antibodies to streptavidin. Given the incidence of antibody responses to murine antibodies from 8 to 33% in similar patients (23–25) and an incidence of only 1% against the chimeric rituximab antibody according to the package insert, the high incidence of antibody responses to streptavidin in NHL patients has reinforced our view that pretargeting approaches need to be developed with the same concern for immunogenicity of the agents as we have come to appreciate for directly radiolabeled antibodies. Because bsAb can be composed of the humanized counterparts of each of their murine antibody components, it is more likely that the bsAb will be less immunogenic than a construct containing streptavidin or avidin.

One chemically conjugated bsAb (Fab′ × Fab′) prepared from hMN-14 and m734 (26) was initially used for pretargeting evaluation of delivering ^{99m}Tc/^{188}Re-labeled peptides, but more recently, we have focused on the development of a series of bsAb-pretargeting systems that uses an antibody (m679) directed against HSG (27). Such pretargeting methods offer considerable flexibility in the types of effectors that can be used because the antibody binds to the HSG hapten and not to the effector moiety (*e.g.*, DTPA). Thus, a variety of effectors can be incorporated into a basic HSG-containing core peptide, thereby allowing for many targetable substances. Initial studies with two chemically linked bsAbs (mMu-9 × m679 and hMN-14 × m679) in human colon cancer xenografts suggest that both could be applicable for imaging with ^{99m}Tc or ^{111}In or for therapy with ^{90}Y or ^{177}Lu (28). Pilot studies with a ^{90}Y-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″ *N*‴-tetraacetic acid-di-HSG peptide have also shown positive antitumor effects in experimental animals (29).

These promising pretargeting results have encouraged us to embark on a program to develop recombinant bsAbs derived from variable domains of m679 and hMN-14. Recombinantly produced bispecific binding proteins are very important for a variety of reasons. Certainly, the potential for improved manufacturing of a homogenous protein is essential but perhaps even more significant is that recombinant proteins can be designed in a number of configurations, including constructs that would have multiple binding sites for the intended targets, whether it is the tumor antigen or the hapten. We have shown that a chemically prepared bsAb from hMN-14 and m679 with bivalency to the tumor antigen could improve the amount and longevity of effector binding in the tumor (30). However, it is desirable to additionally optimize the pharmacokinetic properties of such multivalent constructs (*e.g.*, by reducing their molecular size) because we would especially prefer to develop the approach without the need for a clearing step that is used for streptavidin-biotin pretargeting systems (4). Herein, we describe our initial efforts to produce two bispecific diabodies (31, 32) and two bispecific trivalent binding proteins in *E. coli* and show that such recombinant bsAbs can have a targeting advantage over the traditionally prepared chemical conjugates.

Materials and Methods

Molecular Cloning. Each of the constructs was engineered using standard molecular biology methods. All restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Benzonase was obtained from Novagen (Madison, WI). All oligonucleotides were synthesized with an Applied Biosystems 392 DNA/RNA synthesizer. PCR reactions

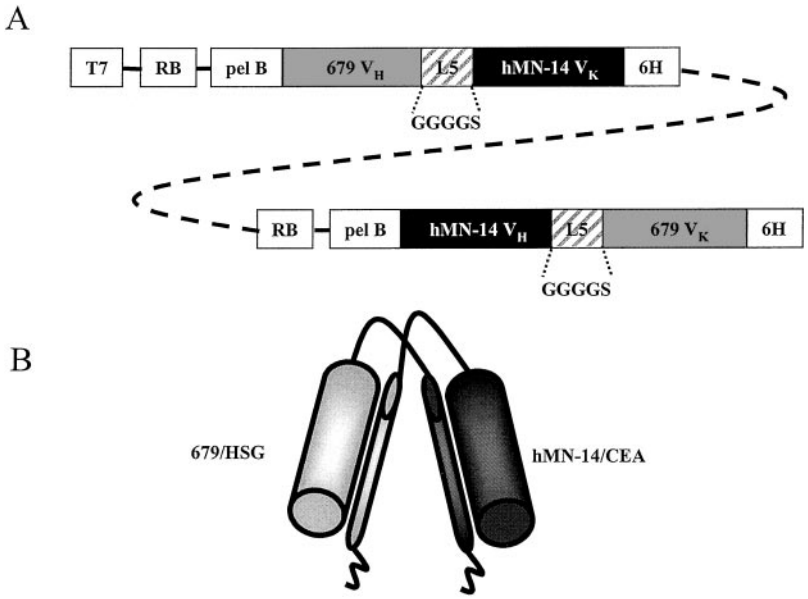

Fig. 1 Schematic representation of the *E. coli* expression cassette (*A*) and synthesized polypeptides that form BS1.5 or BS1.5H bispecific diabodies (*B*). *A*, the expression cassette in the pET-ER vector, which codes for a single RNA message generated from T7 RNA polymerase via the T7 promoter. This message contains two ribosomal binding sites (RB) and the coding sequences for the two heterologous polypeptides. *B*, drawing of a BS1.5 (or BS1.5H) diabody formed from the pairing of the heterologous polypeptides [679V_H(G_4S)hMN-14V_K and hMN-14V_H(G_4S)679V_K] and possessing one binding site each for CEA and HSG.

were performed using Amplitaq polymerase (Applied Biosystems, Foster City, CA) and a Perkin-Elmer GeneAmp PCR system 9600. Some subcloning procedures used pGem3Z vector (Promega, Madison, WI). Each of the final constructs was in a modified vector (pET-ER) that was generated by the addition of a second multiple cloning site into pET26b vector (Novagen) to enable dicistronic expression in *E. coli*. Two complementary oligonucleotides were ligated into the *Blp*I restriction site of pET26b to generate the pET-ER vector.

The hMN-14 V_H and V_K sequences were amplified by PCR from a vector that was constructed for expressing hMN-14 Fab′ (33). The V_H and V_K domains of m679 were amplified by PCR from a plasmid containing the cDNA sequences of m679 heavy and light chains (Qu *et al.*, unpublished data). A DNA sequence encoding five amino acids (GGGGS) was appended to the 3′-end of m679 V_H and hMN-14 V_H during PCR. Humanized versions of m679V_H and V_K were generated by PCR-based mutagenesis. The strategy used for humanization was to retain all Complimentary determining region amino acid sequences and any framework residues known to interact with CDR regions. Amino acid residues of mouse framework regions that are not represented in the database of human frameworks were replaced with the most common amino acid found in human sequences at that position. A total of 13 amino acid substitutions was made to convert m679 to h679.

The coding sequences of V_H domains were ligated to those of V_K domains via *Bam*HI restriction sites, and the resulting V_H-V_K sequences were inserted into *Nco*I/*Xho*I restriction sites of pET-ER vector. The final assembly of the dicistronic expression cassettes in pET-ER for the synthesis of BS1.5 is shown in Fig. 1. For BS1.5H, humanized 679-V domains were used. The final expression cassettes for the trivalent bispecific BS6 and BS8 antibodies are shown in Figs. 2 and 3, respectively. Overlapping synthetic oligonucleotides constituting the L16a and L16b linkers were inserted into the constructs via restriction sites.

Production and Characterization of Recombinant bsAb. Chemically competent *E. coli* BL21-pLysS cells (Novagen) were transformed with the expression plasmids for BS1.5, BS1.5H, BS6, or BS8 following the manufacturer's recommendations. Transformants were plated overnight at 37°C on Lennox L agar plates supplemented with kanamycin sulfate (100 μg/ml) and chloramphenicol (34 μg/ml). Transformed colonies were used to inoculate shake cultures of Difco 2xYT broth (Becton Dickinson, Sparks, MD) supplemented with kanamycin sulfate (100 μg/ml) and chloramphenicol (34 μg/ml). Cultures were shaken at 37°C to an $A_{600\ nm}$ of 1.6–1.8. An equal volume of room temperature 2xYT media supplemented with antibiotics and 0.8 M sucrose was added to the cultures, which were then transferred to 20°C. After 30-min shaking at 20°C, expression was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 40 μM, and the incubation continued at 20°C for 15–18 h. Bacteria were pelleted by centrifugation at 10,000 × *g*. The cell pellets were frozen and thawed then resuspended in lysis buffer [2% Triton X-100, 300 mM NaCl, 10 mM imidazole, 5 mM $MgSO_4$, 25 units/ml benzonase, and 50 mM NaH_2PO_4 (pH 8.0)] using an amount equal to 1% of the culture volume. The suspension was homogenized by sonication and clarified by centrifugation at 40,000 × *g*. The soluble extracts were loaded onto Ni-NTA agarose (Qiagen, Inc., Valencia, CA) columns using ~1.0 ml of Ni-NTA resin/liter culture. Columns were washed with a buffer containing 20 mM imidazole and eluted with 250 mM imidazole. The Ni-NTA eluate was loaded directly onto a Q-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) anion exchange column, and the flow through fraction containing the products was collected. The yield of BS1.5 and BS1.5H were ~0.5 mg/liter culture. The yields of BS6 and BS8 were ~50 and 20 μg/liter, respectively.

Size exclusion HPLC was performed on a Beckman System Gold Model 116 with a Bio-Sil SEC 250 column (Bio-Rad,

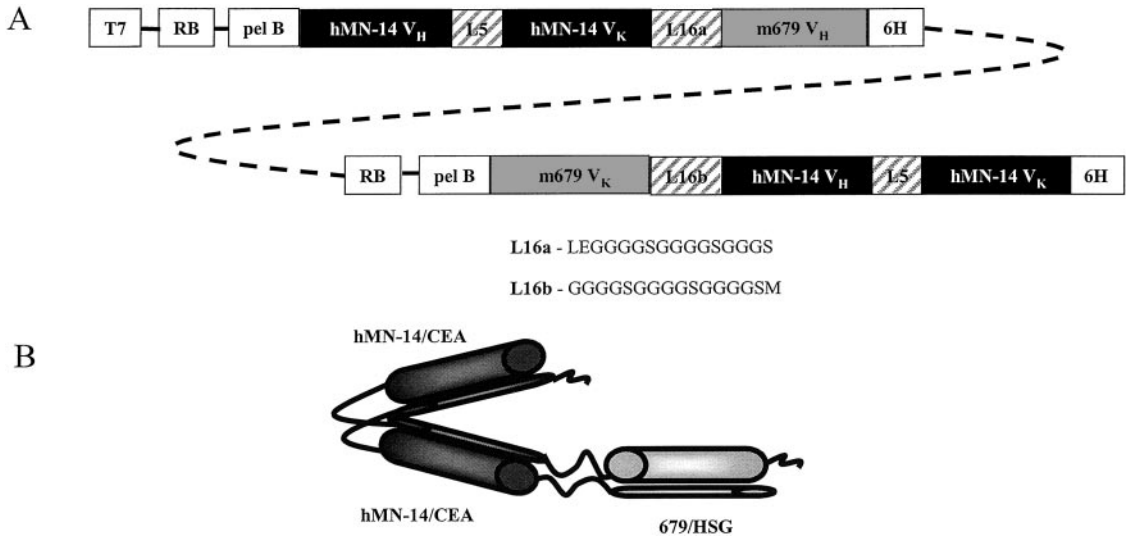

Fig. 2 Schematic representation of the *E. coli* expression cassette (*A*) and synthesized polypeptides that form the trivalent bispecific BS6 (*B*). *A*, the expression cassette in the pET-ER vector, which codes for a single RNA message generated from T7 RNA polymerase via the T7 promoter. This message contains two ribosomal binding sites (RB) and the coding sequences for the two heterologous polypeptides. *B*, drawing of the BS6 trivalent bispecific protein structure possessing one binding site for HSG and two binding sites for CEA.

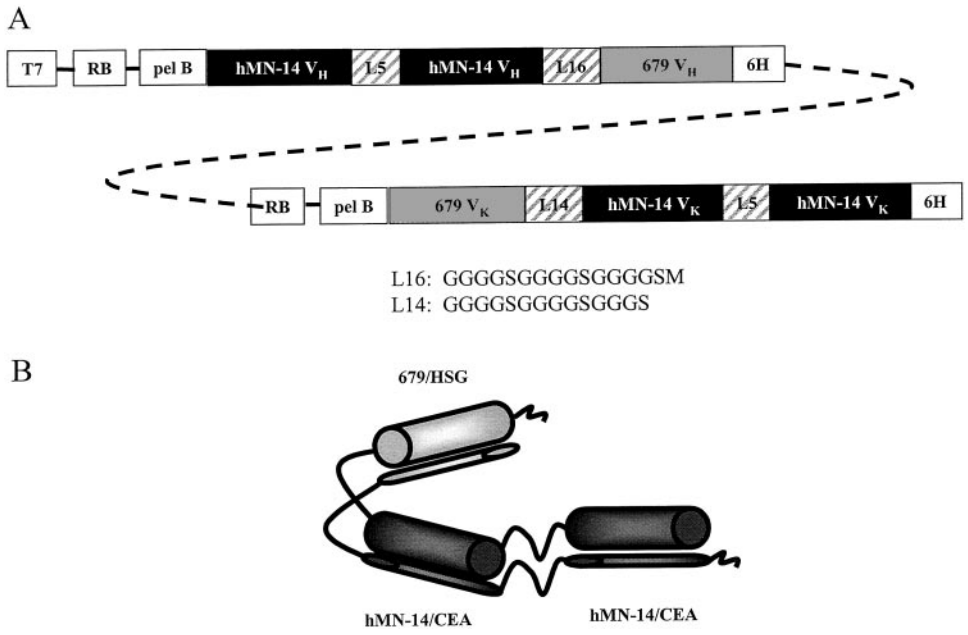

Fig. 3 Schematic representation of the *E. coli* expression cassette (*A*) and synthesized polypeptides that form the trivalent bispecific BS8 (*B*). *A*, the expression cassette in the pET-ER vector, which codes for a single RNA message generated from T7 RNA polymerase via the T7 promoter. This message contains two ribosomal binding sites (RB) and the coding sequences for the two heterologous polypeptides. *B*, drawing of the BS8 trivalent bispecific protein structure possessing one binding site for HSG and two binding sites for CEA.

Richmond, CA). A variety of standards were used to calibrate the column, including hMN-14 IgG (M_r ~150,000), hMN-14 Fab′ (M_r ~50,000) modified with *N*-ethylmaleimide, hMN-14 F(ab′)$_2$ (M_r ~100,000), hMN-14 diabody (M_r 53,000), and hMN-14 triabody (M_r 80,000).

BIAcore Analysis. A BIAcoreX system (Biacore, Inc., Piscataway, NJ) was used to analyze the binding properties of the bispecific proteins. The binding kinetics of BS1.5 was evaluated using a low-density HSG-coupled sensor chip prepared according to the manufacturer's instructions. Binding sensorgrams were obtained for BS1.5 and hMN-14 × m679 at concentrations from 0 to 54 nM, and the resulting data were analyzed with the BiaEvaluation software using 1:1 Langmuir binding model. The bispecific binding properties of each agent were analyzed by BIAcore with a high-density HSG-coupled biosensor chip prepared according to the manufacturer's instructions. Fifty μl of BS1.5, BS1.5H, BS6, or BS8 (each at 1.0 μg/ml) were prebound to the sensor chip before a 100 μl-injection of WI2 (20 μg/ml), a rat monoclonal anti-idiotype antibody that is highly specific for hMN-14 (34), was applied.

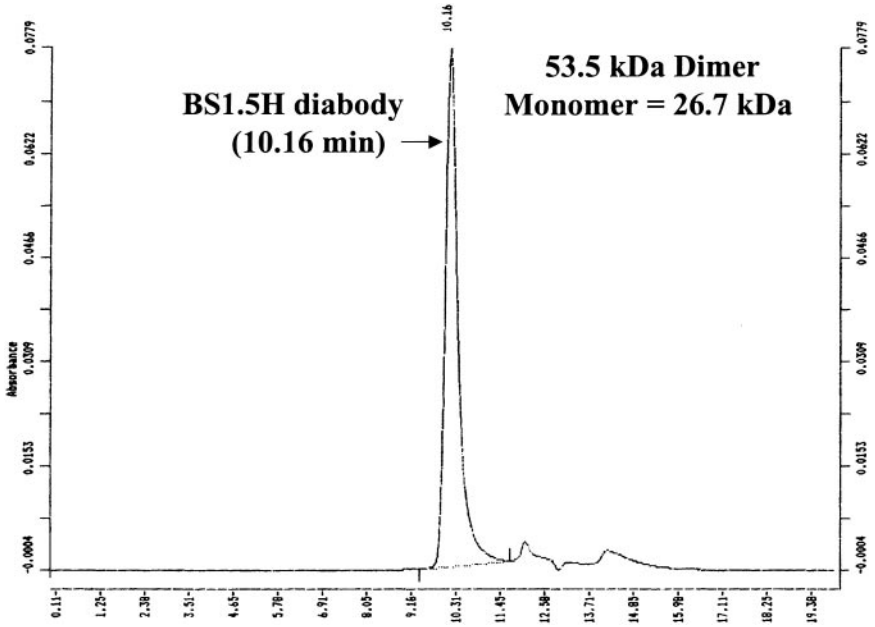

Fig. 4 Size exclusion HPLC profile of BS1.5H after purification by immobilized metal affinity chromatography and Q-Sepharose chromatography.

Soluble CEA (Scripps Laboratories, La Jolla, CA) was also used in place of WI2 and gave similar results.

ELISA. Competitive ELISA experiments were performed to demonstrate the binding of BS1.5 or BS1.5H to CEA via its hMN-14 binding site. Microtiter plates were coated at 0.5 μg/well with soluble CEA. BS1.5, BS1.5H, or hMN-14 × m679, at concentrations ranging from 4 to 500 nM, was allowed to compete for CEA binding with horseradish peroxidase-conjugated hMN-14 IgG (1.0 nM). The absorbance was measured at 415 nm with a microtiter reader after addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid tablets (Sigma, St. Louis, MO).

Determination of Immunoreactivity. The immunoreactivity of BS1.5, BS1.5H or hMN-14 × m679 for CEA was evaluated on size exclusion HPLC by measuring the fraction of a radioiodinated sample that is shifted, in the presence of excess CEA, toward shorter retention time as a result of binding to CEA. The ability of BS1.5, BS1.5H or hMN-14 × m679 to bind to the radiolabeled peptide was similarly demonstrated on size exclusion HPLC by noting the shift of the radiolabeled peptide toward shorter retention time upon adding the bispecific construct.

***In Vivo* Studies.** Female NCr nude mice weighing 19–21 g (Taconic, Germantown, NY) bearing s.c. CEA-positive GW-39 (35) human colon cancer xenografts were injected i.v. with ^{131}I-labeled BS1.5 prepared by an iodogen method (36) to assess its biodistribution. For pretargeting studies, 8 μg of BS1.5 or BS1.5H (each trace labeled with ^{125}I or ^{131}I) were injected i.v. and then 8 and 15 h later (BS1.5 and BS1.5H, respectively), IMP-241 (15 pmol) labeled with 10 μCi of ^{111}In according to previously published methods (28) was injected. The biodistribution of ^{111}In-IMP-241 was examined at 3 and 24 h after its injection.

Results

The dicistronic expression cassette shown in Fig. 1 for BS1.5 encodes two heterologous polypeptides that are designed to pair with each other and form one functional binding site each for CEA and HSG via domain swapping (37). A similar dicistronic expression cassette was constructed for BS1.5H by substituting the variable domains of m679 in the dicistronic expression cassette of BS1.5 with the humanized versions (E. Rossi *et al.*, unpublished data). We created dicistronic expression cassettes for two novel types of trivalent bispecific constructs by judiciously linking three variable domains with spacers of appropriate length into each of the two cistrons, as exemplified by BS6 (Fig. 2) and BS8 (Fig. 3). Each of the four proteins was obtained from the soluble fraction and purified by immobilized metal affinity chromatography using Ni-NTA followed by Q-Sepharose anion exchange chromatography. As shown in Fig. 4, BS1.5H yielded a single peak on SE-HPLC and the observed retention time was consistent with the expected molecular size of M_r ~54,000, indicating the formation of a dimer. A similar HPLC purity and retention time were obtained for BS1.5 (data not shown). The retention time observed for BS6 or BS8 was also consistent with the expected molecular size of M_r ~81,000, indicating the formation of a dimer.

Additional characterization was provided by BIAcore experiments to demonstrate bispecific binding properties, as shown in Fig. 5 for BS1.5H and BS6. Each protein bound tightly to immobilized HSG on a sensor chip. The HSG-bound proteins were able to capture subsequently added CEA or WI2, demonstrating that they can simultaneously bind both antigens. If the WI2 binding is allowed to reach saturation, the stoichiometry of the binding can be determined. The additional increase in RU resulting from WI2 binding was compared with the initial RU increase of the bispecific protein upon binding to the HSG

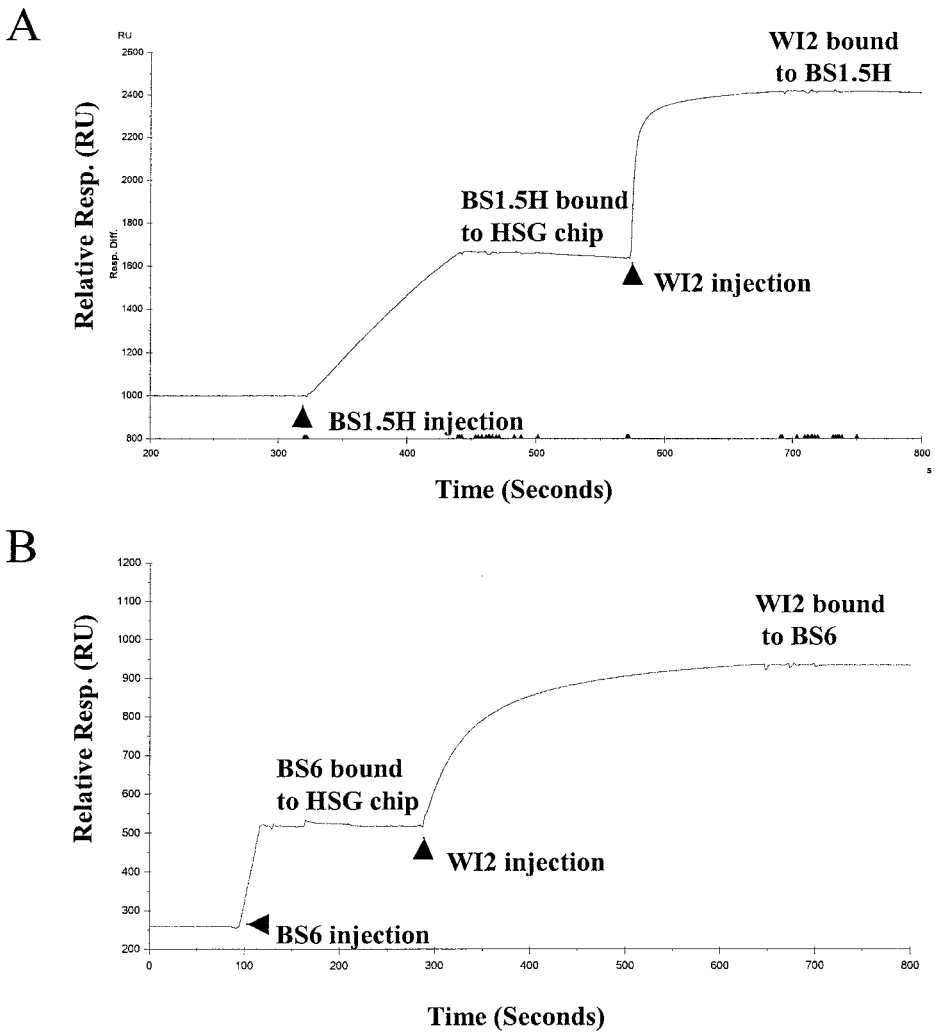

Fig. 5 BIAcore sensorgrams showing simultaneous HSG and CEA binding for BS1.5H (*A*) and BS6 (*B*). BS1.5H or BS6 (~50 ng) was loaded on a high-density HSG-coupled sensor chip, and then WI2 (2 μg) was allowed to bind to the immobilized bispecific protein. *Arrows* indicate injection times.

sensor chip. As each increase in RU level is directly proportional to the mass bound, the WI2:bsAb molar binding ratio can be calculated using the formula $(RU_{WI2}/RU_{bsAb}) \times (MW_{bsAb}/MW_{WI2})$. Because WI2 is bivalent, 1 mol of WI2 is expected to bind two moles of BS1.5 or BS1.5H, either of which is monovalent for CEA; therefore, the theoretical maximum WI2:bsAb molar binding ratio should be 0.5. The experimentally determined molar binding ratios for BS1.5, BS1.5H, and hMN-14 × m679 were found to approach the theoretical maximum of 0.5 (Table 1). BS6 and BS8 were designed to be bivalent for CEA (and monovalent for HSG) and as such should bind WI2 with a 1:1 molar ratio. Indeed, the experimentally determined molar binding ratios of WI2 to BS6 or BS8 were found to be between 0.8 and 0.9, also approaching the theoretical maximum of 1.0 (Table 1). These BIAcore results combined with the HPLC data demonstrate that each of the four constructs yields a protein that has the structure and function as designed.

The HSG binding parameters of BS1.5 and hMN-14 × m679 were analyzed by BIAcore and summarized in Table 2. The dissociation constant (K_d) for HSG as determined for BS1.5 was similar to that of hMN-14 × m679. The affinity of BS1.5H for HSG was also found to be similar to that of BS1.5 based on a qualitative analysis on BIAcore (data not shown), indicating that humanization of m679 variable domains did not measurably alter the HSG binding affinity. As shown in Fig. 6, competitive ELISA experiments demonstrated that BS1.5 and hMN-14 x m679 had a similar binding affinity (K_d, ~10 nM) for CEA. In a separate competitive ELISA experiment, the binding affinity of BS1.5H for CEA was measured and was also found to be ~10 nM.

Table 1 Experimental and theoretical molar binding ratios of WI2 to bsAb

	Experimental molar ratio	Theoretical maximum ratio
BS1.5	0.4	0.5
BS1.5H	0.43–0.47	0.5
hMN-14×m679	0.46	0.5
BS6	0.84–0.87	1.0
BS8	0.79–0.85	1.0

The biodistribution of BS1.5 was evaluated in nude mice

Table 2 HSG binding parameters determined by BIAcore

	K_d nM	k_{on} $M^{-1}s^{-1}$	koff s^{-1}	Molecular weight M_r (kDa)
BS1.5	2.5	4.05e5	1.01e-3	53.5
hMN-14×m679	1.55	3.59e5	5.56e-4	100

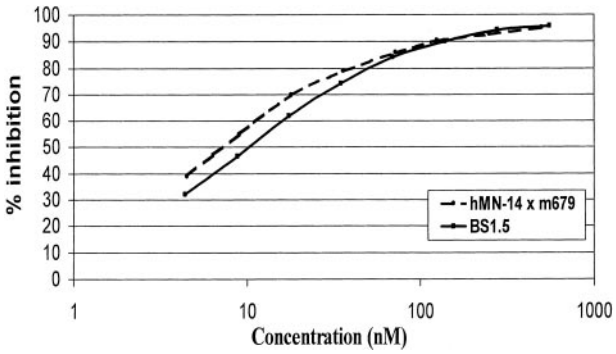

Fig. 6 Graphical representation of the results of a competitive ELISA experiment. Horseradish peroxidase-conjugated hMN-14 IgG (1 nM) was mixed with either BS1.5 or hMN-14 × m679 at concentrations ranging from 4 to 500 nM before incubation in CEA-coated (0.5 μg/well) ELISA wells. The percentage of inhibition is plotted against concentration (nM) of sample.

bearing GW-39 tumor, using a sample trace labeled with radioactive iodine (immunoreactivity, ~90%). BS1.5 cleared rapidly from the blood (Fig. 7), starting with 15.9 ± 1.3% ID/g in the blood at 1 h, 1.46 ± 0.37% ID/g by 8 h, and 0.26 ± 0.03% ID/g by 24 h after injection. Tumor uptake at 1 h was 7.6 ± 2.1% ID/g, which decreased to 4.7 ± 0.7 and 2.8 ± 0.4% ID/g at 8 and 24 h, respectively, after its injection. In the kidneys (data not shown in Fig. 7), uptake was first measured at 61.5 ± 3.2% ID/g at 1 h, but this rapidly decreased to 2.5 ± 0.2 and 0.6 ± 0.16% ID/g at 8 and 24 h after injection, reflecting the rapid filtration and lack of any significant readsorption of the radioiodine by the kidneys. A comparable biodistribution was found for BS1.5H, showing 3.1 ± 0.7% ID/g in tumor with 0.36 ± 0.07% ID/g in the blood at 18 h after its injection.

The utility of BS1.5 and BS1.5H for tumor pretargeting was evaluated in GW-39 tumor-bearing mice using ^{111}In-labeled IMP-241. Studies were initially performed with BS1.5 using several intervals between the peptide and the bsAb to determine an optimal timing for pretargeting. On the basis of our prior work (26, 28, 29), a mol injection ratio of 10:1 bsAb:peptide was used for both BS1.5 and BS1.5H. As shown in Table 3, with the peptide given just 8 h after injecting BS1.5, tumor uptake of the peptide was 10.3 ± 2.7% ID/g at 3 h after the peptide was injected, with tumor/blood ratios exceeding 150. This uptake was ~100 times higher than that seen for the peptide alone. Tumor uptake decreased to 6.3 ± 2.2% ID/g 24 h after the peptide was given, but T/NT ratios maintained a very high level. As more time elapsed before the peptide was administered, tumor uptake of the peptide was less than that seen with an 8-h interval, and no additional improvements in T/NT ratios were observed with intervals > 8 h. An ANOVA analysis

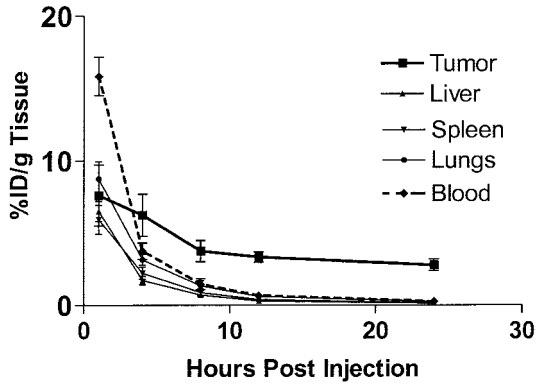

Fig. 7 Biodistribution of ^{131}I-labeled BS1.5 in nude mice bearing GW-39 human colonic tumor xenografts (tumor sizes averaged from 0.15 to 0.3 g). *Bars* represent SD of mean (n = 4 to 5 mice).

comparing the necropsy data for tumor uptake at 3 h for the three different intervals revealed there was a significant difference among these groups (P = 0.012), with the 8-h interval significantly higher than the other two intervals. Using a single-tail t test to examine differences in the tumor uptake data at 24 h revealed that the 8- and 12-h intervals were not significantly different (P = 0.3), but the 8 and the 12-h interval were significantly higher than the 24-h interval (8 *versus* 24 h, P = 0.01; 12 *versus* 24 h, P = 0.03). Thus, of the intervals tested, the 8-h one was the best for achieving the highest tumor accretion. However, given the remarkably high T/NT ratios, it may have been possible to administer the peptide even earlier. A similar study was performed for BS1.5H to determine the best interval for administering the peptide, and a 15-h interval was selected.

The pretargeting results using BS1.5 were compared with those obtained with hMN-14 × m679 and BS1.5H. For hMN-14 × m679, a 24-h interval and a 10:1 bsAb/peptide injection mol ratio were determined to be optimal for pretargeting (28). At 24 h, hMN-14 × m679 typically had ~0.8% ID/g in the blood with ~3.0% ID/g in the tumor, both comparable with results observed with BS1.5 at 8 h or BS1.5H at 15 h. Under these conditions, the concentration of ^{111}In-IMP-241 in the tumor was similar for each of the three pretargeting agents. However, the radioactivity in all normal organs was higher in mice pretargeted with hMN-14 × m679 (Fig. 8). Therefore, the T/NT ratios (Table 4) were superior for the two bispecific diabodies.

Discussion

Slow blood clearance of directly radiolabeled intact IgG results in high background activity, which reduces the amount of radioactivity that can be administered. Although this has not limited the success of treatment for radiosensitive hematological malignancies, solid tumors will require higher amounts of radioactivity to achieve significant antitumor effects (2, 3). Antibody fragments such as Fab or F(ab′)$_2$ clear from the blood more rapidly than whole IgG, which improves target-to-background ratios but at the expense of lower uptake of the radiolabeled antibody in the tumor when compared with whole IgG (38). Although antibody fragments have been used for imaging

Table 3 Biodistribution of ^{111}In-IMP-241 in nude mice bearing GW-39 tumors (size between 0.15 and 0.3 g) necropsied at either 3 or 24 h after peptide injection

Mice were given an i.v. injection of BS1.5 either 8, 12, or 24 h preceding the peptide injection (n = 4–5 animals/group).

	Peptide alone	BS1.5 administered with the following clearance times before ^{111}In-IMP-241					
		8 h		12 h		24 h	
	3 h	3 h	24 h	3 h	24 h	3 h	24 h
Tumor	0.14 ± 0.06	10.3 ± 2.7	6.3 ± 2.2	6.3 ± 1.6	5.5 ± 1.9	6.3 ± 1.2	3.1 ± 1.4
Liver	0.08 ± 0.009	0.15 ± 0.02	0.13 ± 0.01	0.08 ± 0.009	0.09 ± 0.006	0.13 ± 0.01	0.08 ± 0.004
Spleen	0.03 ± 0.003	0.07 ± 0.009	0.05 ± 0.007	0.04 ± 0.005	0.09 ± 0.05	0.06 ± 0.006	0.04 ± 0.01
Kidney	2.2 ± 0.2	3.6 ± 0.8	2.3 ± 0.5	2.0 ± 0.5	1.1 ± 0.1	3.1 ± 0.6	1.9 ± 0.3
Lungs	0.09 ± 0.03	0.21 ± 0.01	0.05 ± 0.008	0.28 ± 0.23	0.08 ± 0.04	0.12 ± 0.03	0.03 ± 0.001
Blood	0.01 ± 0.001	0.06 ± 0.005	0.01 ± 0.001	0.04 ± 009	0.03 ± 0.02	0.03 ± 0.01	0.002 ± 0.001

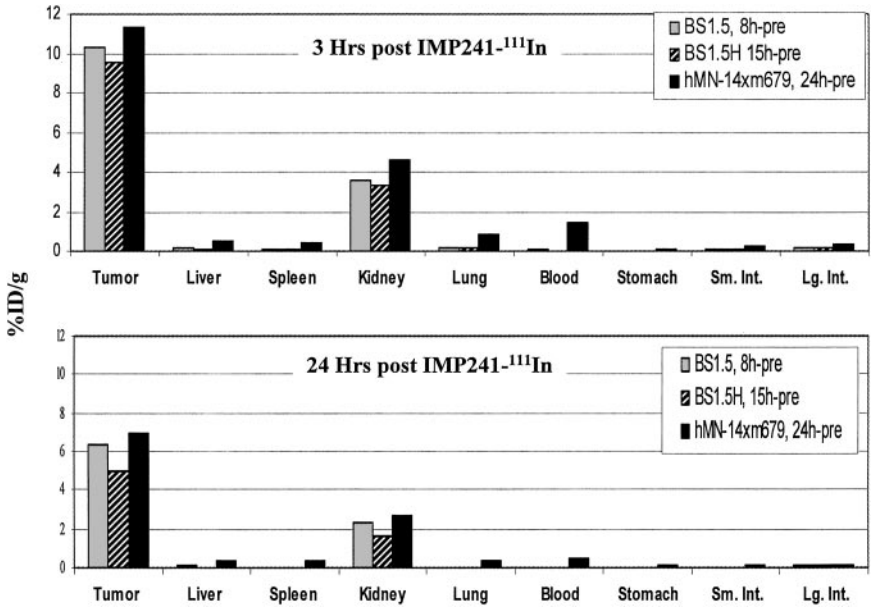

Fig. 8 Biodistribution of ^{111}In-IMP-241 in tumor-bearing mice pretargeted with BS1.5 (■), BS1.5H (▨), or hMN-14xm679 (■). GW-39 tumor-bearing nude mice, injected with BS1.5, BS1.5H, or hMN-14xm679, were pretargeted for 8, 15, and 24 h, respectively. ^{111}In-IMP-241 was injected after pretargeting. Radioactivity in tissues was measured at 3 and 24 h after injection of ^{111}In-IMP-241 and shown as % ID/g in the figure.

Table 4 Comparison of T/NT ratios[a] at 3 h after ^{111}In-IMP-241

Time elapsed before peptide injection	BS1.5 8 h	BS1.5H 15 h	hMN-14×m679 24 h
Liver	68.4 (±18.8)	120 (±36)	22.2 (±6.3)
Spleen	157 (±46)	181 (±58)	27.8 (±5.9)
Kidney	2.9 (±0.7)	2.98 (±1.1)	2.5 (±0.5)
Lung	50.9 (±15.6)	48.6 (±19.3)	14.1 (±2.8)
Blood	167 (±34.7)	284 (±50.6)	8.1 (±2.1)
Stomach	488 (±242)	530 (±291)	103 (±15.2)
Small intestine	116 (±55)	235 (±138.7)	53.4 (±14.4)
Large intestine	59.4 (±27.6)	61.2 (±33.2)	37.4 (±9.2)

[a] SD is shown in parenthesis.

(39) and some preclinical studies have shown them effective therapeutically (40), there has only been limited interest in developing radiolabeled antibody fragments for therapy. The primary reason for this is the high retention of radiolabeled antibody fragments in the kidneys, predominantly when radiometals are used in contrast to radioiodine (41, 42). Renal uptake of radiometal-labeled antibody fragments can be reduced by the preadministration of lysine (43), but even this method fails to produce high tumor/kidney ratios.

Well-designed pretargeting approaches can address these issues. In pretargeting systems, the radionuclide is attached to a rapidly clearing agent. The two most commonly used agents reported to date have been biotin, radiolabeled with ^{111}In/^{90}Y for the streptavidin pretargeting approach and a di-DTPA-Tyr-Lys peptide radiolabeled with ^{131}I or ^{111}In for the bsAb pretargeting method. In each instance, these radiolabeled compounds clear rapidly from the body, primarily by renal excretion. Renal readsorption is less than that seen with radiometal-labeled Fab′ fragments, and thus, tumor/kidney ratios exceed 1 within just a few hours of the radiolabeled biotin or peptide injection. Despite the rapid clearance from the blood and body, various investigators have shown that with pretargeting it is possible to localize a sizable fraction of the radiolabeled effector in the tumor. Indeed, interest in using pretargeting for therapy was boosted considerably by the study of Axworthy *et al.* (6), which showed the % ID/g of a radiolabeled biotin localized in a tumor could equal that of a directly radiolabeled antibody. This opened the

possibility that not only could pretargeting procedures produce better T/NT ratios earlier than a directly radiolabeled antibody but that the amount of radioactivity delivered to the tumor could be the same. Over time, investigators have shown improved therapeutic benefit of pretargeting compared with direct targeting, and clinically promising antitumor effects have been reported in glioma (44) and in NHL.[4] These encouraging results warrant additional consideration being given to pretargeting for therapy.

We have been investigating a novel pretargeting system that involves the use of m679, an antibody that reacts with the HSG hapten (27). This system, using chemically conjugated bsAb, has already been shown to have excellent targeting properties with proven antitumor activity in preclinical testing. Although very useful for proof of principle, if pretargeting procedures are to advance to clinical applications, additional considerations regarding the pretargeting agent must be given. First, the ability to manufacture large quantities of a bsAb in a reproducible manner is essential. Secondly, preclinical testing has suggested that a construct with two binding sites for the primary tumor target will improve the uptake and retention of the bsAb at the tumor site compared with a bsAb that has only a single binding site for the tumor antigen (29). Thirdly, the bsAb must also have certain pharmacokinetic properties if the procedure is to be simplified by not requiring a separate clearing step (29). Preparation of bsAb using recombinant DNA technology would fulfill all of these requirements. For example, these proteins could be produced in bacteria or yeast in large-scale fermentors to provide large quantities of the bsAb. Microbial fermentation media is inexpensive compared with mammalian cell culture media. Even with modest yields of 20 mg/liter from *E. coli* fermentation, production is markedly less costly as compared with monoclonal antibodies produced at much higher levels in bioreactors with mammalian cells. For bsAb prepared by chemical conjugation, two different cell lines will need to be grown to make the bsAb, whereas a single cell line would produce the desired recombinant bsAb. Using molecular engineering, a suitable bsAb may be designed with binding and pharmacokinetic properties that are equal or better than those achievable by a chemically linked bsAb. In addition, a recombinant bsAb can also be made less or nonimmunogenic by adopting an all-human immunoglobulin sequence such as BS1.5H.

The characterization of BS1.5 and BS1.5H included the determination of purity by SE-HPLC and SDS-PAGE, the determination of molecular size by SE-HPLC, the determination of binding affinity for CEA by competitive ELISA, the determination of binding affinity for HSG by BIAcore, and the determination of binding valency for CEA by BIAcore. BIAcore binding experiments gave K_d values for HSG of 2.5 and 1.55 nM for BS1.5 and hMN-14 × m679, respectively. These values are essentially equivalent because the difference is within the experimental error range of the assay. Competitive-binding studies by ELISA suggested that the CEA-binding properties of BS1.5 and hMN-14 × m679 were also similar. *In vivo* targeting studies further confirmed this, but BS1.5 predictably had faster clearance from the blood and tissues because it is ~2-fold smaller in molecular size than hMN-14 × m679, which actually cleared considerably faster in mice than would be expected for a protein of M_r ~100,000. We have shown that the chimeric nature of a bsAb like hMN-14 × m679 (*i.e.*, composed of one part humanized and one part mouse) causes such a type of construct to clear more quickly in mice than a conjugate composed fully of murine Fab′ fragments (26). However, in humans, the same type of chimeric construct has been shown to clear from the blood more like an $F(ab')_2$, as would be expected (17). Certainly, the smaller bispecific diabody such as BS1.5 would still be expected to clear in humans faster than a chemical conjugate with the size of an $F(ab')_2$. This gives BS1.5 a pharmacokinetic advantage and would shorten the interval required for administration of the peptide, potentially avoiding the need for developing a clearing step. Pretargeting studies in tumor-bearing mice showed that BS1.5 produced similar tumor uptake but with higher T/NT ratios than hMN-14 × m679, illustrating how the rapid clearance combined with comparable binding strength for CEA and HSG of BS1.5 can be used as an advantage for pretargeting. For all tissues except the kidney, the ratios obtained for pretargeting with BS1.5 were >5-fold greater than those with hMN-14 × m679. The higher activity in kidneys is attributed to renal clearance of the radiolabeled peptides as designed. Although the tumor/kidney ratio is significantly lower than for other organs, the ratio of 3:1 is still favorable for imaging and therapeutic applications. Because bone marrow toxicity often determines the maximal-tolerated dose for a radiolabeled antibody, the superior tumor/blood ratios with this pretargeting procedure should enable considerable escalation of injected activity and potentially improve therapeutic efficacy. Furthermore, the smaller molecular size may facilitate penetration and give a more uniform distribution of the antibody and peptide in the tumor.

In addition to reducing the radioactivity concentration and retention in the blood and improving the distribution of radioactivity in the tumor, another way to increase therapeutic efficacy is to increase the retention of the bsAb/peptide in the tumor. Two trivalent bispecific proteins, as exemplified by BS6 and BS8, were designed to accomplish this. This type of construct contains one HSG and two CEA binding groups and has a size that is 20% smaller than the hMN-14 × m679 chemical conjugate. Bivalent CEA binding should increase tumor retention and possibly tumor uptake as well. Furthermore, a protein of this size (M_r ~81,000) may prove to be ideal for a pretargeting agent because it would be expected to make several passes at the tumor, allowing for higher uptake, and yet still have favorable clearance rates. Unfortunately, the expression levels of BS6 or BS8 in *E. coli* were too low to allow these agents to be studied in experimental animals. However, we have succeeded recently in producing similar trivalent bispecific proteins in yeast with much higher yields, and preliminary biodistribution studies under pretargeting conditions are very encouraging (H. Karacay *et al.*, unpublished results).

The design of BS1.5 or BS1.5H was based on the well-

[4] R. M. Sharkey, H. Karacay, H. Richel, W. McBride, E. A. Rossi, K. Chang, D. Yeldell, G. L. Griffiths, H. J. Hansen, and D. M. Goldenberg. Optimizing bispecific antibody pretargeting for use in radioimmunotherapy, submitted for publication.

established diabody technology (32, 45). The use of the five-amino acid peptide linker (GGGGS) was intended to invoke the predominant formation of diabodies rather than other possible structural forms such as triabodies (46). Biochemical analysis clearly demonstrated that BS1.5 and BS1.5H almost exclusively formed diabodies.

BS6 and BS8 are novel scFv-based structures formed by the noncovalent association of three V_H-V_K pairs. With BS8, all three V_H domains are contained on a single polypeptide whereas the three V_K domains form the second polypeptide chain. BS6 has one chain consisting of two V_H and one V_K and another chain consisting of two V_K and one V_H. With both, the domains are arranged in a specific antiparallel manner to facilitate pairing of complementary domains without steric constraint. The design should allow for the formation of only one type of dimer—the proper heterodimer—because like domains are not expected to associate with each other. V_H domains that are not paired with V_L domains are unstable and insoluble because of exposure of hydrophobic surfaces and, as a result, unpaired polypeptides will degrade and/or precipitate. BS6 and BS8 were characterized by SE-HPLC and SDS-PAGE for purity, SE-HPLC for molecular size, and BIAcore for binding valency to CEA. After extensive purification, the HPLC profiles of both BS6 and BS8 showed a major peak with a retention time consistent with a protein of M_r ~80,000. Using BIAcore, we have also determined that BS6 and BS8 indeed possessed two functional binding sites for CEA. Furthermore, the fact that BS6 and BS8 bind HSG-coupled sensor chips with high affinity demonstrates that they contain a properly paired 679 F_v. A trivalent bispecific protein that contains two binding sites for a target tumor and one binding site for a hapten has considerable potential for use in a variety of applications, especially as a pretargeting agent for the AES.

In conclusion, our initial experience using a recombinant bsAb such as BS1.5 as a pretargeting agent is very encouraging. With the prospects for additional improvements in both the design and production of recombinant bsAb, we believe that the bsAb-pretargeting approach will significantly enhance the therapeutic index for antibody-mediated delivery of radionuclides and possibly also cytotoxic drugs.

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