In Vivo Biodistribution of a Humanized Anti-Lewis Y Monoclonal Antibody (hu3S193) in MCF-7 Xenografted BALB/c Nude Mice

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ABSTRACT

The biodistribution characteristics of a humanized anti-Lewis Y antibody (hu3S193) radiolabeled to three radioisotopes, 125I, 111In, and 90Y, were examined in a BALB/c nude mouse xenograft model of breast cancer. The immunoreactivity of both 125I- and 111In-bound hu3S193 exceeded 50% and was 20% for 90Y. In vivo, labeled antibody was shown by gamma camera imaging and immunohistochemical and autoradiographic techniques to localize to Lewis Y-expressing breast xenografts with minimal normal tissue uptake. Maximal radioisotope uptake peaked at 48 h for all three isotopes; however, the percentage of injected dose/gram and tumor retention were greater for 111In- and 90Y-bound antibody than for 125I-bound antibody. Although immunoreactivity of 111In- and 125I-labeled hu3S193 in serum was stable over a 5-day period, the amount of unlabeled 111In in serum was lower than 125I, which together with higher tumor uptake indicates better retention of 111In-labeled hu3S193 and catabolites within the tumor cells. Superior tumor uptake and retention of 111In-labeled hu3S193 and similar blood clearance compared with 125I-labeled hu3S193, suggest that radiomimetics are the preferred radioisotope for this antibody-antigen system. Humanized 3S193 is a promising new construct for the targeting and potential therapy of Lewis Y-expressing tumors.

INTRODUCTION

Despite advances in the treatment of breast cancer in terms of new chemotherapeutic and hormonal agents, the median survival of patients with advanced breast cancer has remained virtually constant over the last 30 years, and recurrent breast cancer remains incurable (1). New treatment strategies are required if there is to be any impact on the current plateau in survival. Conventional radiotherapy and chemotherapy are delivered to both normal and neoplastic cells, relying upon the enhanced sensitivity of rapidly dividing cancer cells to achieve preferential killing (2). In contrast, mAbs2 can be directed or “targeted” against tumor associated antigens which may be expressed on the surface of the tumor cells (3).

A number of tumor antigens have been shown to have high expression on malignant breast cancer cells, including HER2/neu, MUC, carcinomaembryonic antigen, and Le Y (3). The Le Y antigen is a family member of the blood group-related difucosylated oligosaccharides with chemical structure Fucα1→2Galβ1→4[Fucα1→3] GlcNAcβ1→R(4). They are associated with 60–90% of human carcinomas of epithelial cell origin including breast, colon, gastric, and lung cancer (5–7). The high frequency of Le Y-expressing tumors, high density of Le Y on tumor cell surface, and relatively homogeneous expression in primary and metastatic lesions have led to its selection as an antigenic target.

3S193 is a mAb produced using standard hybridoma techniques following immunization of BALB/c nude mice with Le Y-expressing MCF-7 breast cancer cells. The murine antibody has been shown to have high specificity for Le Y and reacted strongly in rosetting assays and cytotoxicity tests with Le Y-expressing cells (8). The use of murine mAbs, however, is limited by their immunogenicity. Murine antibodies are recognized as foreign, and the immune system mounts a classical humoral immune response producing HAMAs. Although clinical HAMA requires rechallenge to the same antibody, serum sickness reactions can occur after the first or repeated infusions and are usually unrelated to dose or rate of administration and are manifested principally by myalgias and arthralgias (9). A further limitation of many murine antibodies is their inability to harness the effector and complement response of the human immune system as effectively as humanized antibodies, depending upon the isotype of the antibody (10).

CDR grafting is one method of humanizing antibodies, combining murine antigen binding regions (CDR) with human V-region framework determinants. CDR grafting aims to make the surfaces of the antibody appear as fully human as possible, but retain the murine antigen-binding packaging and interface reactions determining high-affinity binding (11). 3S193 has been humanized by CDR grafting, and the reactivity for Le Y has been confirmed to be similar to the murine version, and with retained potent effector function of complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (8, 12). hu3S193 has only 3–5% murine residues in the antibody variable domain, markedly reducing the potential for an immune response in planned human trials.

The role of conventional radiotherapy in metastatic breast cancer has historically been limited to local palliation. Given that breast cancer is a radiation-sensitive disease (2), targeted delivery of radiation of effective dose to all metastatic sites using mAbs would be a novel and potentially useful therapeutic modality. Radiouclides are attractive agents for selective targeting to tumors by chemical conjugation to antitumor antibodies. The emitted radiation targeted to tumors can be used to kill tumor cells (therapy) or to detect primary or metastatic tumor (imaging), depending on the isotope selected. Considerations in selecting a radionuclide include type of emission, its range, tumor dose rate and total tumor dose, biological half-life, labeling efficiency, and stability. To be effective, the link between the antibody and the radionuclide must be stable in vivo, and the labeling procedure must not alter the biodistribution or binding characteristics of the antibody. This study explores such properties of hu3S193 bound to 125I, 111In, and 90Y.

MATERIALS AND METHODS

mAbs. The generation of murine 3S193 using standard hybridoma technique after immunization of BALB/c mice with Le Y-expressing MCF-7 breast cancer cells and its subsequent humanization has been described previously (8, 12). hu3S193, a CDR grafted version of 3S193, was produced by Scotgen (Aberdeen, Scotland) in conjunction with the Ludwig Institute for Cancer Research. hu3S193 used for the experiments outlined was obtained from both the New York Branch, and the Biological Production Facility of the Melbourne Branch of the Ludwig Institute for Cancer Research. huA33, a humanized...
antibody directed against a novel antigen found in >95% of colorectal cancers (13), was supplied by the New York Branch of the Ludwig Institute and used as a subclass-specific control.

**Cell Lines.** MCF-7, a Le- expressing human breast adenocarcinoma cell line originally derived from the pleural effusion of a 69-year-old woman with estrogen receptor-positive metastatic breast cancer (14), was obtained from the American Type Culture Collection (Rockville, MD). SW1222, a Le-negative human colon cancer cell line, was a gift from the tumor cell bank of the New York Branch of the Ludwig Institute and was used as a control cell line.

Cells were grown in 175-cm² plastic flasks (Nunc; Nunc, Roskilde, Denmark) and maintained in log-phase growth in RPMI 1640 (Trace Biosciences, Sydney, Australia) supplemented with 10% (MCF-7) or 5% (SW1222) FCS (MultiSer; Trace Biosciences, Sydney, Australia), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 mM sodium pyruvate, 2 µM glutamine, and essential amino acids. Cells were cultured at 37°C in a 5% CO₂ incubator (Forma Scientific, Inc., Marietta, Ohio) and passed with 0.05% EDTA/PBS (BDH Chemicals, Merck, Melbourne, Australia). Cell viability in all experiments, as determined by trypan blue exclusion, exceeded 90%.

**Radiolabeling and Quality Assurance.** hu3S193 and control antibody (huA33) were labeled with three isotopes, 125I, 111In, and 90Y. Isotopes were obtained from DuPont (Life Science Products, Boston, MA). Radiiodination was performed using a modification of a previously published chloramine-T reaction (15) using a 2-fold molar excess of chloramine-T (Merck, Darmstadt, Germany) over antibody, dissolved in 0.5 M potassium phosphate buffer (pH 7). After a brief 2 min incubation period, the reaction was stopped by adding a 5-fold excess of sodium metabisulfite, again dissolved in a 0.5 M phosphate (pH 4.5) and 0.9% saline/10 mM NaOH solution as solvents. In EDTA, acetate, followed by 10 mM EDTA. The radiolabeled mixture was purified by a desalting column (P6DG; Bio-Rad, Sydney, Australia) was treated with 10 mM sodium bicarbonate and 100-fold unlabeled hu3S193 was added. After 45 min incubation, the cells were washed and counted as described above for Lindmo assay. The IR fraction was taken into account in calculating the amount of free, reactive antibody ([100 – % bound]/[100 × total antibody × IR fraction], and specific binding (nM total antibody × % bound) was graphed against specific binding/reactive free. The association constant was determined from the negative slope of the line. The number of hu3S193 molecules bound per cell was derived by: [(X-intercept of Scatchard plot (nM/1000) × (6.023 × 10²³)]/number of cells used in the assay (12 × 10⁶).

**Animal Model.** In vivo tissue biodistribution studies were performed in female athymic BALB/c nude mice, 5–6 weeks of age, homozygous for the nu/nu allele, bred by the SPF Facility, University of South Australia (Adelaide, Australia). Mice were maintained in autoclaved microisolator cages housed in a positive pressure containment rack (Thoren Caging Systems, Inc., Hazelton, PA). To establish MCF-7 human breast xenografts, mice were supplemented with exogenous estrogen (20, 21). After light ethane anesthesia, a 60-day slow release estrogen pellet (0.72 mg estradiol/pellet; Innovative Research of America, Sarasota, FL) was inserted using aseptic techniques into a small s.c. pocket of the back.

**Biodistribution Studies.** Two biodistribution studies were performed. In the first study, 41 mice were injected i.v. via the retro-orbital plexus with hu3S193 labeled with 5 µCi of both 125I and 111In CHX-A-DTPA-hu3S193 (total, 10 µg of antibody and 100 µCi of radioactivity) suspended in 150 µl of sterile media. Groups of four to five mice, with a mean (± SD) tumor volume of 647 ± 252, were sacrificed by cervical dislocation after light ethane anesthetic at 4, 24, 48, 72, 96, 120, 168, 240, and 360 h after injection of radiolabeled antibody. Mice were bled via cardiac puncture, and blood was collected into heparinized tubes. Tumors and organs [skin, liver, spleen, intestine, stomach, kidneys, brain, bone (femur), lungs, and heart] were immediately removed, blotted dry, and weighed (Sartorius Basic Balance, Ratingen Germany). All samples were counted in a dual chamber gamma scintillation counter (Cobra II, Auto-gamma; Packard Instruments) using a dual tracer program with standard windows set for each isotope, 15–75 Kev for 125I and 140–430 Kev for 111In. Standards prepared from the injected material were counted each time, with tissues and tumors enabling calculations to be corrected for the physical decay of the isotopes. Results of labeled antibody distribution over time were expressed as the % ID/g ([cpm tissue sample/cpm cpm standard] × 100/weight in grams) and as tumor: blood ratios. A second biodistribution study was also performed to assess the distribution...
and localization of 99mTc-labeled antibody. Twenty μCi of 99mTc-CHX-A-DTPA-hu3S193 were injected into the retro-orbital plexus of 25 mice bearing MCF-7 xenografts. One to four mice were sacrificed at time points 4, 24, 48, 72, 120, 168, 240, 336, 408, and 504 h after injection and blood and tumors removed. At the 72-h time point, spleen, bone, liver, and kidney were also excised. % ID/g was calculated as described above.

**Statistical Analysis.** Paired Student t tests were performed on the different isotope biodistribution time points in each study and individual organ uptakes to assess any differences observed for statistical significance.

**Pharmacokinetics.** Pharmacokinetics were determined for 125I and 111In radioconjugates using a curve-fitting program (SAAM II; University of Washington, Seattle, WA) and assuming a two-compartmental model.

**In Vivo Antibody Stability.** The stability of the iodinated model and iodium label in vivo was assessed by the amount of free radionuclide present over time, as determined by ITLC. Blood collected from mice at each time point into heparinized tubes was pooled and spun at 1000 × g for 15 min to obtain plasma. Two μl of each sample were spotted onto the origin of two standard ITLC strips. Strips were developed, and the percentage of free iodine and iodium was calculated as described previously.

A single point immunoreactivity assay was also performed on each pooled plasma sample to determine the in vivo stability of the antibody in terms of binding ability. An aliquot of plasma, volume adjusted to standardize for % ID/g, was added to 12 × 106 MCF-7 cells and incubated for 45 min at room temperature. Excess unbound antibody was removed at the end of the incubation period by washing twice with medium, and radioactivity in the washed pellet was measured with a gamma counter. An equal volume of blood as that added to the assay was kept as a standard, and binding of blood to cells was calculated as a percentage of antibody present in the standard.

**Imaging.** To enable qualitative comparison of radiolabeled-antibody localization over time, a single mouse from the 125I/111In co-labeled antibody study was anesthetized on four separate occasions. At time points 4, 24, 72, and 120 h, the animal received i.p. injections of 0.2 ml of solution containing 18.7 mg/ml tribromoethanol dissolved in 12.5 μl of isopentyl alcohol and 1 ml of sterile water for injection. Images were obtained using a dual head gamma camera (“Biad”; Trionix Research Laboratories, Twinsbury, OH) linked to a Unix computer system with photopeaks set at 173 KeV and 247 KeV with 20% windows. A medium energy collimator was used to acquire images attributable predominantly to 111In, with negligible contribution from 125I. Ten-min images were obtained at each time point. Images were acquired in a 256 × 256-bit matrix, and a standard of known activity was included in the field of view.

**Autoradiography.** Immediately after sacrifice, an MCF-7 tumor from one mouse at time points 4, 24, 48, 72, and 120 h in the 125I/111In biodistribution study was excised, frozen, and sectioned on a cryomicrotome (Zeis Microm HM 5000, Melbourne, Australia). Sequential 5-μm sections were used for direct autoradiography and for H&E staining. Tissue sections were placed on silane-coated glass slides and allowed to dry. Glass slides were placed face down in contact with emulsion of Hyperfilm-MP film (Amersham Life Science, Little Chalfont, United Kingdom) or standard radiography film. The glass slides and film were enclosed in a cassette and stored at ~70°C. An image intensifier (Hyperscreen; Amersham) was used for Hyperfilm samples and was located directly behind and in contact with the film (23). Slides and film were taken up at varying time intervals, and film was developed in a standard automatic film processor. It was not possible to distinguish between 125I and 111In using the method described.

**Immunohistochemistry.** Immunohistochemistry was performed to confirm the presence and distribution of LeY antigen in tumor xenografts and to examine normal murine tissues for LeY antigen expression. Xenografts and normal kidney, liver, lung, spleen, stomach, duodenum, jejunum, ileum, and large bowel were surgically removed, embedded in Tissue-Tek OCT Compound (Diagnostic Division, Elkhart, IN), and cut at 5-μm thickness with a cryomicrotome. Sections were fixed with acetone at 4°C for 10 min, and nonspecific binding was blocked using a commercial protein blocking agent (Lipshaw Immunon, Pittsburgh, PA). Five μg of hu3S193 diluted in 1% BSA-PBS were applied to sections and incubated at room temperature for 1 h (primary antibody), rinsed with PBS, and then the secondary antibody (biotinylated IgG1 goat antihuman 1:300; Sigma Immunochromicals) was applied for 30 min. After 10 min immersion in 0.3% peroxidase, streptavidin peroxidase (1:1000) was applied for 30 min, and finally the chromagen 3-amin-9 ethyl carbazole (Sigma Chemical Co., St. Louis, MO) for 20 min was used to develop pink coloration to antigen-positive cells. An isotype-matched hulgG1 control antibody (huA33) and no primary antibody controls were used for each tissue or time point analyzed.

**RESULTS**

**Antibody Labeling.** ITLC of radiolabeled antibody prior to injection confirmed 94.5% bound 125I (6.5% free) and >95.5% bound 111In-CHX-A-DTPA. The immunoreactivity of 125I was 51.5% and for 111In, 65%. Kd values for 125I and 111In were 0.71 × 10−7 M−1 and 1.017 × 10−7 M−1, respectively, both within the range expected for a carbohydrate antibody (24). The number of antibody molecules bound per cell for each isotope was 7 × 106 for 125I and 4.62 × 106 for 111In. In the 99mTc-CHX-A-DTPA study, 94.1% of radionuclide was bound to hu3S193; however, immunoreactivity was low at 20%. Scatchard analysis was not performed for 99mTc-labeled antibody.

**Biodistribution.** 125I/111In-labeled biodistribution study results are presented in Table 1. The comparison of %ID/g of 125I- and 111In-labeled hu3S193 in MCF-7 and control tumors, blood, and group size at each time point are detailed. The %ID/g of both isotopes in MCF-7 xenografts peaked at 48 h after antibody injection, with the uptake of 111In-labeled hu3S193 being 3-fold greater than 125I (30.1% versus 10%, respectively) and reaching statistical significance (P = < 0.001). Uptake of hu3S193 within breast tumors was superior for the 111In label at all time points studied, and of note 111In uptake was >3-fold that of 125I at t = 48 h to 360 h inclusive (Fig. 1). The blood levels of both isotopes closely approximated each other throughout the study, declining progressively over time. The tumor:blood ratio for 111In-labeled hu3S193 peaked at 5.15 ± 1.65 at 168 h, compared with 2.83 ± 1.2 at 120 h for 125I.

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>125I %ID/g in MCF-7 tumor (mean ± SD)</th>
<th>125I %ID/g in blood (mean ± SD)</th>
<th>125I %ID/g in SW12222 tumor (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>6.4 ± 1.5</td>
<td>5.5 ± 0.9</td>
<td>25.9 ± 2.9</td>
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<tr>
<td>24</td>
<td>24.4 ± 3.4</td>
<td>9.6 ± 2</td>
<td>15.1 ± 1.4</td>
</tr>
<tr>
<td>48</td>
<td>30.1 ± 4.4</td>
<td>10 ± 3.9</td>
<td>12.2 ± 1.3</td>
</tr>
<tr>
<td>72</td>
<td>23.4 ± 4.8</td>
<td>6.3 ± 0.9</td>
<td>7.6 ± 1.8</td>
</tr>
<tr>
<td>96</td>
<td>24.1 ± 3.3</td>
<td>6.3 ± 1.7</td>
<td>6.0 ± 1.9</td>
</tr>
<tr>
<td>120</td>
<td>22.6 ± 2.5</td>
<td>5.5 ± 2.4</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>168</td>
<td>17.3 ± 4.4</td>
<td>4.4 ± 1.6</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>240</td>
<td>9.5 ± 1.1</td>
<td>2.3 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>360</td>
<td>3.5 ± 1.2</td>
<td>0.8 ± 0.3</td>
<td>1.1 ± 0.5</td>
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</table>

* Data for these mice were calculated. Data are presented as mean ± SD.

* Mice bearing MCF-7 LeY positive and control SW12222 tumors received retro-orbital injections containing 5 μCi of both 125I- and 111In-CHX-A-DTPA-hu3S193 (total, 10 μCi antibody; 10 μCi 99mTc label). Groups of four to five mice were sacrificed at the indicated times. The blood was collected, and tumours, large bowel, muscles, and organs were excised and weighed. Radioactivity was measured.
Comparison to the 111 In-hu3S193 biodistribution study is shown in Fig. 3. 90 Y-labeled antibody uptake in MCF-7 tumors peaked at 22% %ID/g (mean 6 SD) at 48 h, which was significantly higher than 125 I in the kidney (%ID/g 9.6, 6.02, and 1.99, respectively) and in the spleen compared with 111 In (%ID/g 0.001 and 0.038, respectively) and in the spleen compared with 111 In (%ID/g 0.009). Differences in bone marrow uptake between the three isotopes were not statistically significant.

**Pharmacokinetics.** The half-life of each isotope bound to hu3S193 was estimated from the %ID/g of blood obtained over the time points sampled. Assuming a two-compartmental model with a four-parameter fit, the mean T1/2 for 125 I-, 111 In- and 90 Y-labeled radiolabeled hu3S193 in normal murine tissues, MCF-7 tumors, and blood at 48 h (%ID/g 0.087) from peak 111 In tumor uptake. At 72 h, hematopoietic organs and kidneys were removed from four mice. Comparison with the results obtained from the 125 I/111 In biodistribution study at the same time points after antibody injection showed higher uptake of 90 Y in kidney, spleen, and bone marrow (%ID/g 9.6, 6.02, and 1.99, respectively). Differences were statistically significant in the kidney compared with 125 I and 111 In (%ID/g 0.001 and 0.038, respectively) and in the spleen compared with 111 In (%ID/g 0.009). Differences in bone marrow uptake between the three isotopes were not statistically significant.

**Table 2** Biodistribution of 90 Y CHX-A'-DTPA labeled hu3S193 in MCF-7 xenografted BALB/c nude mice

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>n°</th>
<th>%ID/g in MCF-7 tumor (mean ± SD)</th>
<th>%ID/g in blood (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>8.92 ± 0.93</td>
<td>30.45 ± 2.48</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>13.26 ± 1.05</td>
<td>14.46 ± 2.88</td>
</tr>
<tr>
<td>48</td>
<td>3</td>
<td>22.47 ± 4.95</td>
<td>8.54 ± 2.47</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>22.38 ± 1.87</td>
<td>7.84 ± 2.15</td>
</tr>
<tr>
<td>120</td>
<td>2</td>
<td>18.52 ± 4.4</td>
<td>5.36 ± 0.87</td>
</tr>
<tr>
<td>168</td>
<td>2</td>
<td>18.96 ± 5.93</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>240</td>
<td>4</td>
<td>12.7 ± 2.2</td>
<td>1.4 ± 0.95</td>
</tr>
<tr>
<td>336</td>
<td>2</td>
<td>8.58 ± 2.13</td>
<td>1.45 ± 1.37</td>
</tr>
<tr>
<td>408</td>
<td>1</td>
<td>10.3</td>
<td>0.67</td>
</tr>
<tr>
<td>504</td>
<td>1</td>
<td>9.62</td>
<td>0.54</td>
</tr>
</tbody>
</table>

a Twenty μCi 90 Y-CHX-A'-DTPA-hu3S193 was injected into the retro-orbital plexus of 25 mice bearing MCF-7 xenografts. One to four mice were sacrificed at the indicated times.

b Time points after injection of antibody. Blood and tumors were removed, blotted dry, and weighed.

c Number of mice per time point. Radioactivity was measured.

**Table 2** Biodistribution of 90 Y CHX-A'-DTPA labeled hu3S193 in MCF-7 xenografted BALB/c nude mice

**Fig. 2.** Biodistribution of 125 I- (○) and 111 In-CHX-A'-DTPA (□)-labeled hu3S193 in normal murine tissues, MCF-7 tumors, and blood at 48 h (A) and 72 h (B) after injection of BALB/c nude mice. Data from groups of five mice are expressed as mean %ID/g; bars, SD.
were removed. %ID/g was calculated. Data are expressed as means; bars, SD.

3S193 was 9.3, 7.4, and 7.5 h and mean T_{1/2} \beta 69.3, 69.3, and 79 h, respectively.

In Vivo Stability. In vivo stability was analyzed in the 125I-hu3S193 and 111In-hu3S193 study. The percentage of bound 111In, as determined by ITLC, was >99.5% for all time points up to 7 days at which time 98% bound 111In was present. The percentage of bound 125I was lower at baseline (93.5%) and decreased to 78% at 7 days. Immunoactivity for 111In-hu3S193 and 125I-hu3S913 at t = 0 h, as determined by single-point binding assay, was 65 and 51.5%, respectively, and slowly decreased over time, not dropping markedly until 7 days after injection, at which time the %IR for 111In-hu3S193 was 27.5% and 125I-hu3S913 was 27%.

Imaging. Localization of 111In-hu3S193 in a single BALB/c mouse bearing an MCF-7 and control SW-1222 tumor over time, as determined by gamma camera imaging, is presented in Fig. 4. At 4 h, no tumor localization was evident, with the image showing generalized blood pool activity and slightly increased activity in the central portion of the scan corresponding to the cardiac area. At 24 h, minimal uptake was seen in the control tumor (left side of image), but there was definite localization of antibody to the MCF-7 Le'2-expressing xenograft (right side of image). Over time with decreasing blood pool activity, the uptake in the MCF-7 tumor was more clearly defined. The mouse was sacrificed after the last image was obtained at 120 h. Radioactivity was clearly localized to the MCF-7 tumor, and the tumor:blood ratio was determined to be 6.07:1.

Autoradiography and Immunohistochemistry. The distribution of radioactivity via autoradiography, the location of viable tumor cells using hematoxylin staining, and antigen distribution as determined by immunoperoxidase techniques were compared in an MCF-7 xenograft using hematoxylin staining, and antigen distribution as determined by immunoperoxidase techniques were compared in an MCF-7 xenograft study. The percentage of bound 111In, as determined by single-point binding assay, was 65 and 51.5%, respectively, and slowly decreased over time, not dropping markedly until 7 days after injection, at which time the %IR for 111In-hu3S193 was 27.5% and 125I-hu3S913 was 27%

DISCUSSION

The role of mAbs in the treatment of breast cancer is currently under active investigation, with a number of unmodified antibodies, radio-immunoconjugates, and antibody-chemotherapy conjugates being studied in the laboratory and in clinical trials. Among these is a humanized antibody directed against the p185HER2 growth factor receptor (encoded by the HER-2/neu oncogene, which is overexpressed in 25–30% of all breast cancers (25). Phase II and Phase III studies of this antibody in patients with metastatic breast cancer showed an objective response rate of 12% (25), and higher response rates have been observed when combined with chemotherapy (26). This antibody has been recently approved by the Food and Drug Administration for use in patients with metastatic breast cancer over-expressing the HER-2 receptor.

In addition, a number of other Le' antibodies have been developed and have reached different stages in preclinical evaluation and clinical testing. These include ABL 364 (BR55-2), an IgG3 antibody derived from the BR55-2 hybridoma (7). Murine ABL 364 administered to patients with cytokeratin-positive tumor cells in their marrow was nonspecific staining in the connective tissue stroma but were negative in the tumor itself (Fig. 5D). Radioactivity, assessed by autoradiography, was distributed throughout regions of viable tumor including the central area of viable tumor cells, with little activity in the central necrotic area (Fig. 5A), indicating specific localization of radiolabeled hu3S193 to tumor cells. A similar even distribution of radioactivity throughout viable tumor cells was seen in all other time points studied (results not shown), and given differences in tumor sections, in particular the quantity of necrotic or connective tissue, there was no qualitative difference in distribution of radioisotope over time.

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Fig. 3. The biodistribution, expressed as % ID/g, of 90 Y-hu3S193 in blood (○) and MCF-7 xenografts (□) and 111In-hu3S193 in blood (●) and MCF-7 xenografts (●) from BALB/c nude mice. Results represent a comparison of two separate biodistribution studies with the individual radiolabels. Groups of one to four mice were sacrificed at time points 4, 24, 48, 72, 120, 168, 240, 336, 408, and 504 h after injection, and blood and tumors were removed. %ID/g was calculated. Data are expressed as means; bars, SD.

Fig. 4. Whole body scintigraphic images of radiolabeled 111In-CHX-A'-DTPA-hu3S913 in an athymic BALB/c nude mouse bearing s.c. MCF-7 (breast) tumor in the left flank (right side of image) and SW1222 colon tumor in the right flank (left side of image). Dorsal images were taken at 10 min (A), 24 h (B), 72 h (C), and 120 h (D) after injection of radiolabeled hu3S193, with the mouse supine on the gamma camera. A standard of known activity was included in the field of view and is visible in the upper left quadrant of each image.
shown to reduce or eliminate Le^+ antigen-cytokeratin (+) cells but not cytokeratin (+) cells in Le^-negative patients (27). However, a dose escalation study of murine BR55-2 in patients with metastatic breast cancer reported only minor clinical responses in 28.6% of patients, with HAMA limiting repeated cycles of treatment (28). A Phase I study with the LMB-1 immunotoxin, incorporating the murine anti-Le^+ monoclonal antibody B3 and recombinant Pseudomonas exotoxin, has also been performed in patients bearing solid epithelial tumors. Responses were observed in 5 of 38 patients studied; however, 90% of patients developed neutralizing antibodies against LMB-1 after one cycle of treatment (29).

Studies in nude mice bearing human lung carcinoma xenografts of another anti-Le^+ antibody, BR96, demonstrated that murine and chimeric BR96 were able to prevent or slow growth when administered soon after cell implantation but had only modest effects with staged tumors (22). In contrast, when chimeric BR96 was conjugated to doxorubicin, complete regressions of xenografted human lung, breast, and colon tumors grown s.c. in nude mice were seen (30). In Phase I and II clinical trials of chimeric BR96 (IgG1) conjugated to doxorubicin in patients with Le^-positive advanced stage carcinomas, limited response rates were observed, whereas gastrointestinal toxicity, which improved with implementation of steroid premedication, was demonstrated (31, 32).

We have examined the in vivo biodistribution and labeling stability of the recently humanized anti-Le^+ antibody 3S193, comparing 125I-, 111In-, and 90Y-labeled hu3S193 in an MCF-7 xenografted BALB/c nude mouse model. Excellent and specific tumor uptake was observed with a Le^-negative control tumor, with the peak tumor (MCF-7) uptake for all three isotopes occurring at 48 h. The %ID/g for both 111In and 90Y were significantly higher than 125I. In addition, both 111In and 90Y were retained within xenografts for a longer period of time than 125I. Lower absolute levels of iodine uptake compared with 111In in xenografts have been demonstrated in other studies (13), and this together with differences in tumor retention of radiolabel most likely represents differences in cellular processing of radio-labeled 125I as opposed to both 111In and 90Y. Catabolism of 125I linked to tyrosine results in the generation of [125I]mono-iodotyrosine within lysosomes, which rapidly leaves the cell via cell-mediated transport systems (18, 33, 34). In contrast, endocytosed 111In-DTPA-antibodies are delivered to lysosomes and hydrolyzed by lysosomal enzymes into small molecular weight metabolites that are retained within tumor lysosomes (13, 33, 34).

Cellular processing of 90Y by tumor cells in vitro has been shown in other antibody-antigen systems to be similar to 111In (35) and is also reflected in similarities in distribution in vivo (36). It has been reported that although the pattern of uptake is comparable, superior tumor retention is obtained with the use of 90Y (36), a conclusion that this current study supports.

Within normal tissues, the uptake of 111In and 90Y in liver, spleen, and kidneys was higher than 125I. The higher uptake values in spleen and liver is consistent with known sequestration of radiometals in reticuloendothelial organs (36). This uptake in normal organs is not a consequence of specific targeting attributable to an absence of Le^- antigen expression in these normal tissues of the mouse but is a reflection of blood flow and catabolism of the radio-labeled conjugates. The increased uptake of 111In- and 90Y-labeled antibody compared with 125I in the kidneys reflects differences in metabolism and excretion of radiometals (37). However, the increased renal uptake of 90Y compared with 111In may also reflect the conjugation of 90Y-CHX-A^-DTPA-hu3S193, given the observed low immunoreactivity of the labeling experiment presented, and warrants further investigation using chelating agents of enhanced stability for 90Y immunoconjugates (37). The %ID/g of 90Y in the bone, assumed to represent bone marrow, was approximately double that of 125I and 111In; however, the total amount present was negligible (2% ID/g), and differences were not statistically significant. Other studies have shown that accumulation of 90Y within bone is a concern with evidence that 90Y accumulates within the bone marrow (36) and indicates that 111In may underestimate 90Y bone marrow uptake.

The differences observed between isotopes in radiolabeling techniques and cellular processing were also reflected in the in vivo stability results. The percentage of 125I-bound radioactivity over time, as determined by ITLC, decreased rapidly, consistent with rapid release of free 125I from lysosomes. In contrast, only 2% of free 111In was detected over the same time period, again consistent with current literature suggesting retention of 111In-DTPA-lysine catabolites by tumor lysosomes (13, 33, 34). Retention of antigen binding for up to
5 days in vivo was also observed for both $^{125}$I- and $^{111}$In-labeled hu3S193.

Although promising, clinical responses with unconjugated mAbs in the therapy of solid tumors have been poorer and less consistent than with hematopoietic neoplasms (38–40), and there remains room for improvement. Given the high frequency of epithelial tumors expressing the Le$^a$ antigen and the high density of Le$^a$ on the surface of tumor cells, hu3S193 has potential therapeutic applications. The in vivo studies presented demonstrate that $^{111}$In- and $^{125}$I-radiolabeled hu3S193 have favorable biodistribution characteristics, with high %ID/g uptake of both antibody bound isotopes within Le$^a$-expressing MCF-7 breast xenografts. In contrast, the biological behavior of $^{125}$I-hu3S193, as demonstrated in this report, would indicate that $^{131}$I would not be the preferred isotope for this antibody-antigen system. Tumor localization of $^{111}$In-CHX-A$^-$DTPA hu3S193 was confirmed visually in imaging studies and by autoradiography, demonstrating good penetration of radionuclide to all areas of viable tumor cells. In addition to the direct targeting of the tumor by hu3S193, our previous studies have described the in vitro immune effector functions mediated by the IgG1 Fc portion of hu3S193 (8, 12). The antitumor effect of hu3S193 may be limited in the murine model used in the current study because of restricted murine serum complement activity. The potent in vitro antibody-dependent cellular cytotoxicity of hu3S193 would suggest that, in conjunction with potent complement-dependent cytotoxicity activity in the human, these humoral effects in a clinical setting would augment the therapeutic effects of targeted radiolabeled hu3S193.

hu3S193 has the theoretical advantage of reduced immunogenicity provided by humanization of an antibody, an assumption that has been supported by preliminary data with earlier, fully humanized antibodies (24, 41, 42), allowing multiple courses of treatment that have not been possible with murine or chimeric antibodies. The humanization of 3S913 has yielded a molecule with only 3–5% sequence homology to the parent murine antibody in the immunogenic variable domains. A similar humanization, through CDR grafting, to that of 3S193 has been described for an antihuman CD4 mAb using the same human framework (REI for variable light chain and KOL for variable heavy chain; Ref. 42). Preliminary clinical results in patients have been reported and show a lack of immunogenicity (42, 43).

The humanization strategy we adopted for hu3S193 and the results of clinical studies using mAbs with similar human frameworks provide evidence of the reduced potential for an immune response to hu3S193 in planned clinical trials.

Some Le$^a$ antigen expression was observed in the gastric glands of the stomach and basal intestinal glands of the small bowel of the BALB/c mice used in the current study, a similar distribution pattern to that reported previously in immunohistochemical studies of normal human tissues with other anti-Le$^a$ mAbs (6, 44). However, the current biodistribution study did not demonstrate increased specific targeting to the small bowel of the BALB/c mice (Fig. 2B). The expression of Le$^a$ antigen in normal human tissues (including the GI tract) is of concern in targeting strategies against this antigen; however, the accessibility of normal tissues to anti-Le$^a$ antibodies remains unclear. Results of studies with murine and chimeric antibodies against Le$^a$ suggest that gastric toxicity may be relevant in proposed clinical trials; however, significant toxicity has only been observed at protein doses $\geq$200 mg (31, 32). The optimal protein dose for effective targeting of Le$^a$ tumors (tumor:blood ratios, normal tissue uptake) is yet to be defined with carefully designed biodistribution and biopsy-based trials. In this context, high protein doses of anti-Le$^a$ antibodies reported to cause toxicities may not be required for effective tumor targeting.

The current model has demonstrated the stability of hu3S193, and its characteristics and properties are well suited to beginning trials in the clinic.
TARGETING OF BREAST CANCER XENOGRAPS WITH HUMANIZED 35193


In Vivo Biodistribution of a Humanized Anti-Lewis Y Monoclonal Antibody (hu3S193) in MCF-7 Xenografted BALB/c Nude Mice

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