Radiolabeled Antibody Targeting of the HER-2/neu Oncoprotein

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ABSTRACT

The HER-2/neu oncogene encodes a M, 185,000 transmembrane phosphoglycoprotein which is overexpressed in 25–35% of breast and ovarian neoplasms and portends a poor prognosis. We have studied the feasibility of targeting this oncoprotein, designated p185, with radioiodinated murine monoclonal antibodies (muMABs) 4D5 and 7C2, which recognize distinct epitopes on its extracellular domain. The rates of internalization and catabolism of these antibodies were analyzed by cellular radioimmunomassay and electron microscopy. After binding to NIH3T3 HER-2/neu cells, which show high surface expression of p185, the muMABs were endocytosed via coated pits, routed to lysosomes, and degraded. Approximately 44% of 125I-4D5 and 39% of 125I-7C2 were catabolized by tumor cells after 24 h.

The biodistribution of radiolabeled 4D5 and 7C2 were evaluated in beige/nude mice bearing s.c. NIH3T3 HER-2/neu grafts. A high specificity of localization was seen with tumor:organ ratios of activity generally ranging from 5:1 to 30:1. However, the percentage injected dose of radioactivity per gram of tumor declined sharply from 25% at 24 h to 5% at 120 h postinjection. Treating the animals with 400–700 µCi 125I-4D5 caused a marked inhibition of tumor growth, although no mice were cured. Unlabeled 4D5 had no effect on tumor progression in this model, but administering 400–700 µCi of 131I-DA4-4, an isotype-matched irrelevant muMAB, resulted in an intermediate degree of growth retardation.

Analysis of kinetic blood data and whole-body time-activity curves indicated that the relevant conjugate remained in the body 2–3 times longer than 131I-4D5. Radiiodinated anti-HER-2/neu muMABs are attractive agents for radioimmunodiagnosis and radioimmunotherapy of aggressive HER-2/neu-positive breast and ovarian carcinomas, but effective strategies for retarding intratumoral catabolism may be necessary to optimize their clinical utility.

INTRODUCTION

The neu oncogene was discovered during transfection experiments in which DNA derived from ethylNitrosourea-induced rat neuroglioblastomas was found to induce malignant transformation of NIH3T3 cells (1). The gene was subsequently shown to encode a M, 185,000 transmembrane phosphoglycoprotein bearing extensive structural homology to the epidermal growth factor receptor (2, 3). By probing human genomic and complementary DNA libraries with v-erbB, a viral oncogene encoding a truncated representation of the epidermal growth factor receptor, the human homologue of neu was identified and designated c-erbB-2 or HER-2 (4, 5). The oncogene was also isolated from MAC117, a human mammary carcinoma cell line, where it was found to be amplified 5–10-fold (6).

Several studies have now documented that amplification of the HER-2/neu gene occurs in approximately 25–35% of breast and ovarian adenocarcinomas and is uniformly associated with augmented expression of the oncogene protein p185 (7–9). Moreover, amplification and overexpression of HER-2/neu have been correlated with a poor response to primary therapy and decreased survival (10–13). Since enhanced expression of the HER-2/neu gene product appears to convey a growth advantage on the tumor, it may be possible to modify malignant behavior with monoclonal antibodies which recognize its extracellular domain and reduce surface expression through antigenic modulation. Drebin et al. (14) demonstrated that incubating a neu-transfected cell line, B104-1-1, with an anti-p185 muMAB caused rapid down-regulation of the oncoprotein and loss of anchorage-independent growth, suggesting that expression of p185 was required for maintaining the malignant phenotype. Furthermore, injection of unmodified anti-neu antibodies has been reported to retard growth of HER-2/neu-expressing tumors in at least one model system (15). In addition to any biological effects resulting from direct interaction with the neu protein, anti-HER-2/neu muMABs could also be used to deliver drugs, toxins, or radioisotopes directly to malignant cells. The use of radioiodinated conjugates is particularly appealing, since internalization of the immunoglobulin molecule is not a prerequisite for cytotoxicity. Moreover, tumor cells which are inaccessible to antibody may still be killed by radioimmunoconjugates bound to neighboring cells because of the long path length of the β-particle emitted by 131I.

We have studied the feasibility of targeting the HER-2/neu protein with radioiodinated muMABs 4D5 and 7C2 which recognize distinct epitopes of its extracellular domain. The internalization and catabolism of these antibodies were evaluated by cellular radioimmunomassay and electron microscopy. Their biodistribution, clearance, and therapeutic efficacy were also determined in a murine model utilizing well-established NIH3T3 HER-2/neu tumor grafts.

MATERIALS AND METHODS

Antibodies and Cell Lines. Antibodies 7C2 and 4D5 are murine IgG1 monoclonal antibodies which bind to different epitopes of the extracellular domain of p185 (16). DA4-4 is a murine IgG1 antibody with specificity for the μ chain of human IgM, which was used as an irrelevant control for biodistribution and radioimmunotherapy experiments. SKBR3 and SKOV3 are human breast and ovarian carcinoma cell lines, respectively, which exhibit marked overexpression of p185. NIH3T3 HER-2/neu is a mouse fibroblast cell line transfected with a HER-2/neu complementary DNA insert encoding the entire p185 molecule (17). Transfection was accomplished with a CVN vector containing expression units for mouse dihydrofolate reductase and the bacterial neomycin phosphotransferase gene, which confer resistance to methotrexate and aminoglycoside antibiotics, respectively. In this way, primary transfecants were identified by their selective growth in media containing Geneticin and amplification of the vector accomplished by exposing the cells to increasing concentrations of methotrexate. Cells were maintained in a minimal essential medium supplemented with 12% fetal calf serum, 2 mM glutamine, and 400 mM methotrexate.

Received 6/21/91; accepted 1/23/92.

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1 The abbreviations used are: muMABs, radioiodinated murine monoclonal antibodies; TCA, trichloroacetic acid; PBS, phosphate-buffered saline.
Flow Cytometry. The specificity of binding of muMABs 7C2 and 4D5 to the HER-2/neu gene product was assessed by indirect immunofluorescence. Untransfected 3T3 cells do not express the HER-2/neu gene product and were used as negative controls. 3T3 cells transfected with the HER-2/neu oncogene and SKBr3 cells express high surface densities of the p185 protein and were used as positive cell lines. Cells (5 × 10⁷) of each type were incubated with saturating concentrations of muMABs 4D5, 7C2, or DA-4 for 30 min on ice, washed three times, incubated with a 1:20 dilution of a fluorescein-conjugated, affinity-purified, goat anti-mouse immunoglobulin reagent (TAGO, Inc., Burlingame, CA) for 30 min, washed three times, and then fixed in 1% paraformaldehyde. Cells were analyzed on a FACSSC cell sorter (Becton Dickinson, Inc., Sunnyvale, CA).

Cells were incubated with antibodies, 4D5 and 7C2, were radiolabeled with Na¹²⁵I (Amersham, Arlington Heights, IL) by the Iodo-Gen method as previously described (18). Briefly, 100 µg of antibody were added to 0.5 mCi of Na¹²⁵I in a glass tube coated with 10 µg Iodo-Gen (Pierce, Rockford, IL) for 10 min at room temperature. Free Na¹²⁵I was removed by chromatography on a PD-10 column (Pharmacia, Piscataway, NJ), and eluted fractions were pooled and stored at 4°C. The immunoreactivities of ¹²⁵I-7C2 and ¹²⁵I-4D5 using NIH3T3 HER-2/neu cells were 84% and 79%, respectively, by linear extrapolation to binding at infinite antigen excess (19) (data not shown). The avidities of ¹²⁵I-7C2 and ¹²⁵I-4D5 as determined by Scatchard analysis were 4.48 × 10⁴ L/M and 1.55 × 10⁴ L/M, respectively (data not shown).

NIH3T3 HER-2/neu cells were washed with cold serum-free RPMI 1640, pelleted by centrifugation, and cooled on ice for 45 min. ¹²⁵I-muMABs were then added to cell pellets in a ratio of 0.1 µg protein (20000 cpm)/10⁶ cells and incubated for 1 h. Cells were washed twice with cold RPMI 1640, plated in microtiter wells (1 × 10⁰ cells/well), and warmed to 37°C in a humidified CO₂ incubator. After 0, 1, 4, 10, and 24 h the cultures were assayed for surface membrane-bound, intracellular, and supernatant radioactivity in a Beckman Gamma 5500 counter (Palo Alto, CA) as previously described (20). Cell surface radioactivity was determined by elution of antibody from the cell membrane by a brief (5-min) exposure to a low-pH buffer (pH 1-2).

The cells were subsequently pelleted by centrifugation at 300 × g and removed from the microtiter wells with cotton swabs to determine intracellular cpm. Culture supernatants (0.2 ml) were treated with 0.6 ml 25% TCA to precipitate protein-bound ¹²⁵I released from the cell surface. This was separated from TCA-soluble supernatant radioactivity by centrifugation, and both fractions were assayed in a gamma counter.

Immunoelectron Microscopy. NIH3T3 HER-2/neu cells were grown to confluence in 96-well plates and then incubated with ¹²⁵I-muMABs 7C2 (64 µg/ml) for 45 min at 4°C. The cells were washed twice with cold media, and then a 1:2 dilution of a horseradish peroxidase-conjugated, monovalent, Fab' goat anti-mouse immunoglobulin antiserum was added for an additional 45 min. The plates were washed again and incubated at 37°C for 0, 15, 30, or 240 min and fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate (pH 7.4). Cells were then reacted for 15 min with 3,3'-diaminobenzidine (Sigma, St. Louis, MO) at 0.5 mg/ml in 0.05 M Tris buffer, pH 7.6, containing 0.0009% hydrogen peroxide. After two washes in 0.1 M cacodylate, cells were postfixed in 1% osmium tetroxide and dehydrated in graded ethanol. Cell layers were then bisected, embedded in OCT compound (Miles, Inc., Elkhart, IN) and frozen in a dry ice-ethanol bath. Full-thickness 6-µm cryosections were cut, fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate (pH 7.4), dehydrated in graded ethanol, and air dried.

¹²⁵I-4D5 (or ¹²⁵I-DA-4) was detected by dipping slides in Kodak NTB-2 nuclear emulsion (International Biotechnologies, Inc., New Haven, CT), exposing for 1 week at 4°C, developing in Dektol (Kodak), fixing, and staining. A Nikon Microphot microscope was used to enumerate silver grains and perform photomicrography.

Radioimmunotherapy and Radioimmunoscintigraphy. For therapy experiments, muMABs 4D5 and DA-4 (0.6-1.0 µg) were radioiodinated with Na¹³¹I (6-10 mCi) to specific activities of 7.2-9.3 µCi/µg. Following PD-10 column chromatography, >98% of the ¹³¹I in the resulting radioimmun conjugates was protein bound, as demonstrated by cell- and tissue autoradiography. Groups of 4-7 tumor-bearing mice each received injections of 400 µCi (range, 392-432 µCi) of ¹³¹I-muMABs in 0.6 ml PBS in Experiment 1 (45 µg/mouse) and 700 µCi (range, 667-705 µCi) in Experiment 2 (100 µg/mouse). Additional control groups received either unlabeled muMAB 4D5 (45 µg/mouse in Experiment 1; 100 µg/mouse in Experiment 2) or PBS. Tumors were measured twice weekly in 3 dimensions with a precision caliper, and the mean volumes were plotted versus time to generate growth curves. The percentage increase in tumor size was calculated as:

\[
\% \text{ increase} = \frac{V_t - V_0}{V_0} \times 100
\]

where \(V_t\) is the mean tumor volume on day \(t\) and \(V_0\) is the mean tumor volume on the day of treatment.

At 0, 24, 48, 72, 144, and 216 h the mice were anesthetized and imaged for 10 min with a 400T General Electric gamma camera equipped with a high-energy collimator. The camera was peaked for ¹³¹I using a symmetric window of 38 keV. The activity within each mouse image was determined, and mean values were used to construct whole-body time-activity curves for ¹³¹I-4D5 and ¹³¹I-DA-4. Tumors were outlined as regions of interest with a dedicated ADAC DPS-3300 computer, and the intraregional activity was measured in order to generate tumor time-activity curves. Data were corrected for radioactive decay by serially imaging an ¹³¹I standard. All animals were given potassium iodide as described above.
RESULTS

Antibody Specificity. Indirect immunofluorescence with flow cytometry demonstrated intense, specific binding of the 4D5 and 7C2 antibodies to cell lines known to express high surface antigen densities of the p185 HER-2/neu gene product (3T3/HER-2/neu cells, SKBR3 and SKOV3) but no significant binding to cell lines lacking oncoprotein expression (e.g., untransfected 3T3 cells). Fig. 1 shows typical results from one of five experiments performed (using 3T3/HER-2/neu cells).

Cellular Radioimmunoassays. Internalization and catabolism of $^{125}$I-4D5 and $^{125}$I-7C2 by NIH3T3 HER-2/neu, SKBR3, and SKOV3 cells were evaluated in vitro using a cellular radioimmunoassay. Pulse-labeled cultures were assayed after 0, 1, 4, 10, and 24 h of incubation at 37°C for surface membrane-bound, intracellular, and supernatant radioactivity. Three separate experiments yielded similar results. There was a steady loss of surface cpm from NIH3T3 HER-2/neu cells, with a reciprocal increase in supernatant cpm for both 4D5 and 7C2 (Fig. 2, a and b). Intracellular cpm peaked at 22% after 4 h for 4D5 and 17% after 1 h for 7C2. After 24 h the percentage of total radioactivity remaining cell associated (i.e., cell surface plus intracellular cpm) was 46% and 56% for 4D5 and 7C2, respectively. Conversely, after 24 h 54% and 44% of total culture cpm were found within the supernatant for 4D5 and 7C2, respectively. Supernatants were treated with 25% TCA to distinguish intact $^{125}$I-muMABs (TCA-precipitable) from low-molecular-weight catabolites (TCA-soluble). TCA-soluble cpm progressively increased during the 24-h incubation period for both 4D5 and 7C2, reaching peak values of 44% and 39% of total culture cpm, respectively (Fig. 2, c and d). Hence, approximately 44% of $^{125}$I-4D5 and 39% of $^{125}$I-7C2 were internalized, degraded, and exocytosed by the tumor cells in 24 h. The same pattern of internalization and catabolism was observed when these antibodies were incubated with human breast (SKBR3; Fig. 3) and ovarian (SKOV3; not shown) carcinoma cell lines, which also overexpress the HER-2/neu oncoprotein.

Immunoelectron Microscopy. The endocytic pathway of muMAB 7C2 was studied by immunoelectron microscopy in vitro. NIH3T3 HER-2/neu cells were incubated with saturating concentrations of 7C2 for 30 min at 4°C, washed three times, and then incubated for 30 min with a monovalent, horseradish peroxidase-conjugated Fab' goat anti-mouse immunoglobulin antiserum. Cells were washed again with cold buffer and then warmed to 37°C for 0, 15, 30, or 240 min before processing for electron microscopy as described in "Materials and Methods." At 4°C anti-HER-2/neu antibodies remained circumferentially distributed on the surface of cells (Fig. 4a), but when cells were warmed to 37°C endocytosis rapidly ensued. Within 15–30 min of incubation at 37°C, large numbers of peroxidase-labeled

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Fig. 4. Immunoelectron microscopy documenting endocytosis of anti-HER2/neu antibodies by NIH3T3 cells transfected with the HER-2/neu oncogene. Indirect immunoperoxidase cytochemistry was used to study the internalization of muMAB 7C2 as described in “Materials and Methods.” a, muMAB 7C2 was densely distributed on the surface of NIH3T3 HER-2/neu cells at time 0. Within 15–30 min of incubation at 37°C large numbers of peroxidase-labeled “coated pits” (b, arrows), “coated vesicles,” and uncoated endocytic vesicles (c) were detectable. Large numbers of peroxidase-labeled dense body lysosomes became evident between 30 and 240 min of incubation (d). Bars, 1 μm (a, c, d) or 0.1 μm (b).

“coated pits,” “coated vesicles,” and uncoated endocytic vesicles were detectable (Fig. 4, b and c). Large numbers of peroxidase-labeled dense body lysosomes became evident between 30 and 240 min of incubation (Fig. 4d). Similar results were observed with other anti-HER-2/neu antibodies and other p185-expressing cell lines (e.g., SKBR3; data not shown).

In Vivo Biodistribution. The biodistribution of muMABs 4D5 and 7C2 were evaluated in beige/nude mice bearing s.c. NIH3T3 HER-2/neu tumor grafts. Animals received simultaneous i.p. injections of 10 μg each of 125I-4D5 and 125I-DA4-4. At 6, 18, 24, 48, 72, and 120 h postinjection the mice were sacrificed, and their tumors and organs were harvested, washed, weighed, and assayed for 125I and 127I activity. Specific localization of antibody to tumors was documented in four separate experiments. A representative study is shown in Fig. 5a. The concentration of 125I-4D5 within tumor exceeded that in any of the normal organs at all time points evaluated. The tumor:normal organ ratios of radioactivity ranged from 5:1 to 10:1 and were most favorable 24–72 h postinjection (Table 1). The superior localization of 125I-4D5 within tumors was a result of specific binding to the p185 oncoprotein, as illustrated in Fig. 5b. Up to 8-fold more anti-HER-2/neu muMAB than the irrelevant antibody 125I-DA4-4 accumulated in the tumors. However, the percentage injected dose of radioactivity per gram of tissue of 125I-4D5 decreased precipitously from 25% at 24 h to 5% at 120 h postinjection. The area under the blood activity versus time curve was determined for both conjugates and revealed that 125I-DA4-4 remained in circulation approximately 3 times longer than 125I-4D5 (data not shown). Localization indices for the tumor and normal organs are presented in Table

Table 1 Tumor:normal organ ratios for 125I-4D5

<table>
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<tr>
<th>Organ</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>120</th>
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<td>Blood</td>
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<td>1.47</td>
<td>1.35</td>
<td>1.58</td>
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<tr>
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<td>9.20</td>
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<td>6.36</td>
<td>7.14</td>
<td>5.50</td>
</tr>
<tr>
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<td>6.77</td>
<td>8.29</td>
<td>4.01</td>
</tr>
<tr>
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<td>5.25</td>
<td>5.55</td>
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<tr>
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<td>20.65</td>
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<tr>
<td>Thyroid</td>
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<td>10.81</td>
<td>8.86</td>
<td>9.69</td>
<td>6.93</td>
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2. Specific uptake of $^{131}$I-4D5 within tumors was seen as early as 6 h postinjection (localization index = 2.2) and reached a maximum value of 6.6 at 24 h. The biodistribution of muMAB 7C2 paralleled that of 4D5, with up to 28% injected dose of radioactivity per gram of tumor accumulating within tumors at 36 h, and tumor:normal organ radioactivity ratios of 5:1 to 30:1 (data not shown).

Autoradiography of Tumor Nodules. To assess the heterogeneity of antibody distribution within tumor nodules, tumors from 15 mice in 3 biodistribution experiments were processed for autoradiography 18 h after injection of $^{131}$I-4D5, as described in "Materials and Methods." A heterogeneous distribution of autoradiographic silver grains was observed in tumors, with the heaviest grain densities occurring in perivascular locations (Fig. 6).

Radioimmunotherapy and Imaging. In order to assess the therapeutic efficacy of radioiodinated anti-HER-2/neu muMABs in our animal model, beige/nude mice bearing established NIH3T3 HER-2/neu tumor grafts received i.p. injections of 400 $\mu$Ci of $^{131}$I-4D5 (45 $\mu$g/mouse). Groups of control mice received either 400 $\mu$Ci $^{131}$I-DA4-4, unlabeled 4D5 (45 $\mu$g/mouse), or PBS. Tumor volumes were measured twice weekly, and the mean values were plotted versus time to construct growth curves. No significant difference in tumor growth was observed between animals treated with unlabeled 4D5 and PBS (Fig. 7). However, mice treated with $^{131}$I-4D5 showed marked tumor growth retardation. Animals given $^{131}$I-DA4-4 also experienced tumor growth delays, although the degree of inhibition was significantly less pronounced than in the $^{131}$I-4D5 treated group (Fig. 7). The different regimes were also compared by calculating the percentage increase in tumor size at various days posttreatment, relative to the initial tumor volume. Table 3 demonstrates that $^{131}$I-4D5 was 20-fold more effective than $^{131}$I-DA4-4 in retarding tumor growth 24 days after antibody injection and was 75-fold more efficacious than unlabeled 4D5. Although the effect of $^{131}$I-4D5 was striking, tumors eventually progressed in all animals. Tumor regrowth, which generally occurred 3 weeks after therapy, was not due to the selection of an antigen-negative cell clone, since grafts continued to overexpress the HER-2/neu protein when evaluated by immunohistochemistry (data not shown). Rather, treatment failure most likely resulted from the inability to deliver a sufficiently lethal radiation dose to the tumor. No fatal toxicities were observed in mice treated with 400 $\mu$Ci of $^{131}$I-4D5 or $^{131}$I-DA4-4; all deaths at this dose level were due to tumor progression.

In an attempt to achieve curative tumor ablations, we repeated the radioimmunotherapy experiment using a higher $^{131}$I activity (700 rather than 400 $\mu$Ci/mouse; Fig. 8). This higher dose of $^{131}$I-4D5 caused tumor growth retardation similar in magnitude to that seen in mice given 400 $\mu$Ci of the conjugate (Fig. 7) and did not prevent eventual tumor progression (Fig. 8). Furthermore, increasing the radiation dose of the control reagent, $^{131}$I-DA4-4, to 700 $\mu$Ci increased the nonspecific total body irradiation in recipient mice dramatically, resulting in significant tumor regressions in this control group (Fig. 8). At the higher dose level, fatal hematological toxicity was observed in two of five recipients of the $^{131}$I-DA4-4 reagent but in none of the four mice treated with $^{131}$I-4D5. Hematological parameters reached nadir values 14 days posttherapy and returned to normal by day 21 in surviving mice (data not shown). Two of four mice treated with 700 $\mu$Ci $^{131}$I-4D5 survived >8 weeks, whereas only one of five mice treated with $^{131}$I-DA4-4 survived 8 weeks. None of the 10 mice mice treated with unlabeled 4D5 or PBS survived for 8 weeks.

Serial gamma camera imaging was performed as described in "Materials and Methods" to assess the abilities of $^{131}$I-4D5 and $^{131}$I-DA4-4 to image tumor grafts (Fig. 9). Excellent imaging of HER-2/neu-expressing tumors was reliably achieved using $^{131}$I-4D5, with optimal resolution being obtained between 8 and 72 h after injection of $^{131}$I-4D5. Specific localization of radioactivity to tumors was not observed using $^{131}$I-DA4-4, but mice retained radioactivity longer when receiving injections of this antibody. The biological $t_{1/2}$ of $^{131}$I-DA4-4 was 6.5 days, approximately twice that of $^{131}$I-4D5 (3.3 days; Fig. 10a). Tumor time-activity curves for $^{131}$I-4D5 and $^{131}$I-DA4-4 were generated by serially measuring the activity within a region of interest constructed around the tumor image (Fig. 10b). As anticipated, a higher peak activity within the tumor was achieved with the anti-HER-2/neu conjugate. However, this antibody appeared to be cleared from the tumor more rapidly than the irrelevant conjugate. The protracted clearance of the irrelevant antibody from tumor sites most likely resulted from the lack of specific endocytosis and catabolism of $^{131}$I-DA4-4 by tumor cells, as well as from the increased blood flow to rapidly growing tumors in mice given the control reagent.

DISCUSSION

Breast and ovarian adenocarcinomas account for 36% of all adult female malignancies and cause over 50,000 deaths/year in the United States alone (21). Although considerable progress has been made in treating these diseases, approximately 30% of breast cancer patients and 60% of ovarian cancer patients cannot be cured with current therapeutic approaches. Patients whose tumors amplify and overexpress the HER-2/neu gene have an especially poor prognosis (7) and constitute a group for whom new treatment strategies are needed. One novel approach for such patients might involve therapy with immunonoconjugates targeting the HER-2/neu gene product. We have recently tested an analogous approach in 14 patients with relapsed non-Hodgkin's lymphoma using radioiodinated monoclonal antibodies directed against B-lymphocyte differentiation antigens. Preliminary results have been encouraging; all 14 lymphoma patients had objective responses, including 11 complete responses, with remission durations ranging from 4 to 38+ months (22).

The HER-2/neu oncoprotein is an attractive target for antibody-mediated therapy for several reasons. Overexpression oc-

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Table 2. Localization indices for $^{131}$I-4D5

<table>
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<tr>
<th>Time (h)</th>
<th>Organ</th>
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<th>24</th>
<th>48</th>
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<tr>
<td>Liver</td>
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<td>1.22</td>
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<td>Spleen</td>
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curs in approximately one-third of breast and ovarian cancers but not on normal adult tissues (23). In addition, antigen density appears to be uniform throughout a given tumor, so the antibody could theoretically be distributed to all regions of the malignancy (9). Furthermore, excellent concordance has been observed between the level of expression within primary and metastatic or recurrent lesions (8, 9, 12). Finally, the likelihood of developing an antigen-negative cell clone may be reduced in this setting, since continued expression of p185 appears to be a prerequisite for maintaining the malignant phenotype in cells transformed by HER-2/neu (14).

In this report, we have demonstrated the ability of a radioiodinated anti-HER-2/neu muMAB to retard the growth of HER-2/neu-expressing tumors implanted in immunocompromised mice. Several mechanisms may be responsible for the antitumor effect including down-regulation of p185 expression (24) and radiation-induced genetic damage from $^{131}$I (25–27). Antigenic modulation of the HER-2/neu protein may deprive the cell of an important oncogenic growth signal and increase its susceptibility to certain cytokines such as tumor necrosis factor $\alpha$ (28). Hudziak et al. (28) and Drebin et al. (15, 29) have shown that muMABs 4D5 and 7C2 inhibit the in vitro growth of SKBR3 and other tumor cell lines which overexpress p185. However, cell growth resumed when the antibody was removed from the culture media, suggesting a cytostatic rather than cytotoxic effect. Furthermore, coinjection of unmodified anti-HER-2/neu muMABs and HER-2/neu-expressing tumor lines into nude mice has been reported to cause retardation of tumor growth (15). Such an antitumor effect of unmodified anti-HER-2/neu muMABs was not observed in our experiments, presumably because we delayed muMAB therapy for 10 days until welI established, palpable tumor nodules were apparent and because we did not use repetitive antibody injections, as used by Drebin et al. (15).

In our experiments, the antitumor responses observed were most likely a result of radiation damage induced by $^{131}$I, since unlabeled antibody did not inhibit neoplastic growth at the doses used. Although dramatic inhibition of tumor growth was observed with doses as low as 400 $\mu$Ci $^{131}$I-4D5/mouse, permanent tumor eradication was not achievable even when maximally tolerated doses of radioimmunoconjugate were used (700 $\mu$Ci). At least two mechanisms may have contributed to our inability to permanently eradicate tumors in this model system. First, the inhomogeneous deposition of $^{131}$I-4D5 within tumors demonstrated by autoradiography may have resulted in the sublethal irradiation of tumor cells distant from capillaries, allowing subsequent regrowth of tumors. Second, rapid intratumoral antibody catabolism may have compromised the efficacy of this approach by diminishing the retention of $^{131}$I targeted to tumors by the 4D5 antibody. Cellular radioimmunoassays and immunoelectron microscopy demonstrated prompt internalization of 4D5 after binding to cells, followed by intracellular routing to lysosomes where degradation and deiodination occurred, followed by exocytosis of the radiolabel. Similar studies have recently been published by other workers (30). In vivo biodistribution studies demonstrated that the anti-HER-2/neu conjugate reached a 6–7-fold higher peak concentration within tumors than the irrelevant antibody but was also cleared more rapidly from tumors than the nonbinding, noninternalized $^{131}$I-DA4-4 antibody.

Several strategies could be attempted to improve radiation delivery to HER-2/neu-expressing tumors. A fractionated treatment schedule might permit delivery of a higher cumulative dose of $^{131}$I-4D5 without exceeding the tolerated dose levels. Another strategy could be to use a second generation anti-HER-2/neu antibody, such as the anti-HER-2/neu antibody 7C2, which shows an even greater degree of specificity for HER-2/neu (15, 29). As with 4D5, 7C2 has been shown to inhibit the in vitro growth of SKBR3 and other HER-2/neu-overexpressing tumor cell lines (28). Finally, it may be possible to design a muMAB that is radiolabeled with a more potent radionuclide, such as $^{90}$Y, which has a longer half-life and emits a higher energy radiation than $^{131}$I. Such a muMAB would be expected to have a longer half-life and emit a higher energy radiation than $^{131}$I, which is expected to have a longer half-life and emit a lower energy radiation. In conclusion, the results of this study demonstrate the potential of radiolabeled anti-HER-2/neu muMABs for the treatment of HER-2/neu-expressing tumors.
Table 3 Percentage increase in tumor size

Groups of five to seven beige/nude mice bearing s.c. NIH3T3 HER-2/neu grafts were treated with 400 μCi 131I-4D5, 400 μCi 131I-DA4-4, unlabeled 4D5, or PBS. The mean percentage increase in tumor size ± SE was calculated at 6, 12, 17, and 24 days after treatment.

<table>
<thead>
<tr>
<th>Mean % increase (±SE)</th>
</tr>
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<tbody>
<tr>
<td>Day 6 12 17 24</td>
</tr>
<tr>
<td>131I-4D5 (±35)</td>
</tr>
<tr>
<td>131I-DA4-4 (±87)</td>
</tr>
<tr>
<td>Cold 4D5 (±258)</td>
</tr>
<tr>
<td>PBS (±700)</td>
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</tbody>
</table>

Fig. 8. Escalation of the radioimmunotherapy dose used to treat NIH3T3 HER-2/neu tumors. Groups of four or five beige/nude mice bearing NIH3T3 HER-2/neu tumors received injections of 700 μCi 131I-4D5, 700 μCi 131I-DA4-4, unlabeled 4D5, or PBS. Tumor volumes were measured twice weekly, and the mean values were plotted versus time to generate growth curves.

Fig. 9. Gamma camera image of a mouse bearing a NIH3T3 HER-2/neu tumor (arrow) 6 days after i.p. injection of 400 μCi of 131I-4D5.

Radiation dose to tumors by allowing time for normal tissue recovery (e.g., 300 μCi every 3–4 days for three doses). Second, coadministration of cytokines such as interleukin 2, α-interferon, or tumor necrosis factor may increase the permeability of tumor vessels and allow a more homogeneous distribution of 131I-4D5 within tumors. Third, larger antibody doses may enhance the conjugate’s distribution within the tumor, thereby improving microdosimetry. This supposition is supported by the observation that a protein dose of 10 mg/kg was found to have a greater therapeutic effect than 0.5 or 2.5 mg/kg in a human trial using radiolabeled anti-CD37 and anti-CD20 antibodies to treat B-cell lymphomas (22). Finally, inhibition of intratumoral antibody degradation might prolong the retention of radioiodine by tumor cells sufficiently to eradicate tumors (31, 32). All et al. (33) have doubled the retention of a radiolabeled anti-Thy-1.1 mAb by murine SL2 lymphoma grafts by conjugating the antibody to a radioiodinated, nonmetabolizable carbohydrate ligand (tyramine cellobiose) which remains trapped within lysosomes after internalization. Alternatively, pharmacological agents such as monensin, verapamil, and chloroquine could be used to inhibit antibody catabolism in lysosomes. These drugs have reduced the degradation of radioiodi-
nated anti-HER-2/neu muMAbs in vitro by 97% and increased tumor cell retention of radioactivity by >50% (34) but may be toxic in vivo at the required concentrations.

Although these experiments present promising preliminary evidence supporting the possible utility of radiolabeled anti-HER-2/neu antibodies for imaging and treating human breast and ovarian carcinomas, several limitations of the current studies must be acknowledged. First, the level of HER-2/neu amplification in 3T3/HER-2/neu and SKBR3 cells is higher than that reported for most de novo human cancers. Second, the 7C2 and 4D5 antibodies utilized in these studies do not recognize the murine neu gene product. Therefore, the murine tumor model does not precisely parallel the human situation, where antibodies would also bind to some normal cells expressing low levels of the HER-2/neu protein. Further investigations will be required to determine the optimal dose and administration schedule of radiolabeled anti-HER-2/neu muMAbs and to minimize intratumoral antibody catabolism.

ACKNOWLEDGMENTS

We are grateful for the expert technical assistance of Larry Durack and Janet Clark and wish to thank Drs. Janet Eary and Francis Geissler for their advice.

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Radiolabeled Antibody Targeting of the HER-2/neu Oncoprotein


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