Radioimmunotherapy of Breast Cancer Xenografts with Monoclonal Antibody ICR12 against c-erbB2 p185: Comparison of IodoGen and N-Succinimidyl 4-Methyl-3-(tri-n-butylstannyl)benzoate Radioiodination Methods

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Abstract

C-erbB2 p185 is a proto-oncogene product expressed in 25–30% of human invasive breast cancers that is associated with poor prognosis and resistance to endocrine therapy and chemotherapy. It is minimally expressed in normal adult tissues (M. F. Press et al., Oncogene, 5: 953–962, 1990). For this reason, it is an attractive target for radioimmunotherapy and other antibody-directed therapies. ICR12 is a rat IgG2a monoclonal antibody directed against a protein epitope of the external domain of the c-erbB2 p185. We performed experiments to optimize the direct iodination of ICR12 with 125I using the IodoGen method, and we found impairment of immunoreactive fraction with increasing specific activity. N-Succinimidyl 4-methyl-3-(tri-n-butylstannyl)benzoate (MATE) is a tin ester that can be radioiodinated easily and then coupled to the ε-amino group of lysine residues. This method has been shown to have improved uptake in tumors compared with antibody labeled by direct iodination (P. K. Garg et al., Nucl. Med. Biol., 20: 379–387, 1993). ICR12 could be labeled up to 16 mCi/mg by this technique without loss of immunoreactive fraction. Whole-body retention of MATE-labeled ICR12 was less than IodoGen (P < 0.0001). Radioimmunotherapy experiments in athymic mice bearing established MDA MB 361 human breast cancer xenografts showed growth inhibition for >24 days at a dose of 600 μCi/mouse (P < 0.0001) when labeled by the IodoGen technique, and 12 days using the MATE method (P < 0.0001).

Introduction

Monoclonal antibody ICR12 is a rat IgG2a with high affinity for an external epitope on the protein core of the c-erbB2 p185 proto-oncogene product (1). c-erbB2 p185 is a member of the type 1 tyrosine kinase group of growth factor receptors (also known as HER2/new) that has been characterized as a receptor similar to HER3 and HER4 (c-erbB3 and B4), which bind ligands coded for by the same gene as glial growth factors (2). It is overexpressed in a number of tumors, including breast and ovary, in which it is generally stated to be overexpressed in 20–30% of cases. A great deal of research has been directed to the role of c-erbB2 p185 in the biology of tumor invasion and metastasis; it clearly is a marker of poor prognosis (3, 4) resistance to chemotherapy (5), and resistance to endocrine therapy (6). The expression of c-erbB2 is minimal in normal tissues, and it, therefore, is an attractive target for antibody-directed therapy in those patients who overexpress the antigen in their tumors; these also represent a group of patients who are resistant to conventional therapy and would, therefore, potentially benefit from novel therapeutic approaches.

Radioimmunotherapy is now a well-established experimental principle whereby radiolabeled monoclonal antibodies are delivered i.v. with the aim of specific tumor targeting, hence avoiding the normal tissue damage associated with conventional radiotherapy. We initially assessed the effect of antibody dose on the tumor:blood ratio to see whether there is a maximum dose of antibody that saturates the tumor-associated expression of the antigen, above which further labeled antibody remains in the circulation and causes increased bone marrow irradiation.

We assessed the effect of increasing specific activity (mCi/mg antibody) on the immunoreactive fraction of the ICR12 measured by its ability to remain bound to antigen-coated Sepharose beads and compared two radioiodination methods. The IodoGen technique uses 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril as oxidant to allow in situ electrophilic radiohalogen substitution (7). This reaction occurs predominantly on tyrosine residues throughout the antibody molecule and, therefore, possibly includes the variable region of the antibody, reducing the affinity of the antibody for the antigen. The relative number and reactivity of the tyrosine in the binding region compared with those in the remainder of the molecule may, therefore, determine the extent of the reduction in affinity. An alternative approach using compounds such as MATE has been developed (8) and offers a number of potential advantages over direct iodination: (a) the MATE compound is radioiodinated in the presence of oxidizing agent before the introduction of the antibody and, therefore, spares the antibody from the harsh oxidizing environment associated with direct methods; (b) the antibody is coupled via the ε-amino groups of lysine residues, which for some antibodies may be less plentiful in the variable region; and (c) the in vivo stability of the conjugated protein may be increased because of the postulated resistance of these types of labeled conjugates to monoiiodotyrosine and thyroxine dehalogenases (8–10). We performed kinetic experiments in the same mouse model using ICR12 labeled by the two techniques and compared the ability of the conjugate to localize within the tumors and the residence time of the activity in the tumors. Finally, we performed radioimmunotherapy experiments at increasing doses of 131I to assess the tumor dose response using the two labeling techniques.

Materials and Methods

Monoclonal Antibodies. ICR12 is a rat IgG2a monoclonal antibody that was prepared at the Institute of Cancer Research, (Surrey, United Kingdom). The antibody was raised in rats inoculated with the human breast carcinoma cell line BT474 and the hybridoma line grown in the peritoneal cavity of athymic nude rats (1). The ICR55 monoclonal antibody used to capture the antigen in the immunoreactive fraction estimates was similarly produced and recognizes a different epitope on the external protein portion of the antigen (11). ICR16 is a rat monoclonal antibody directed against the epidermal growth factor receptor raised against HN5 cells, which does not bind to c-erbB2 p185, despite the 60% homology between the receptors (12).

Cells. MDA MB 361, BT474, and SKOV3 cells were cultured as described previously (1).

Antibody Labeling Using IodoGen. ICR12 or ICR16 [284–497 μg in 40–70 μl PBS (pH 7.4; 0.058 mM Na2HPO4, 0.017 mM NaH2PO4, 0.068 mM NaCl)] was added to a polystyrene vial coated with 5 μg of IodoGen (Pierce and

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Warriner Ltd., United Kingdom). Na\(^{131}I\) (1–10 μl; 1 mCi/μl; 25–50 Ci/mg; ICN, Ltd., United Kingdom) was added to 10 μl PBS in a separate vial and left to stand for 5 min. This was then added to the IodoGen tube, vortexed, and left to stand at room temperature for 10 min. Labeling efficiency was assessed by TLC using 10% (w/v) trichloroacetic acid on cellulose (Whatman 31-ET) before separation of the labeled antibody from free radioidiode using a Sephadex G25 column (PD10, Pharmacia UK). Labeling efficiency was typically 85–95%, and final radiochemical purity was >98%. Solutions were prepared for i.v. administration by dilution in 0.9% NaCl to give a final volume of 100 μl/mouse.

**Antibody Labeling Using MATE.** The technique was the same as described previously (9). Briefly, for a typical reaction using 1–10 mCi Na\(^{131}I\), 1–10 μl Na\(^{131}I\) (as above) was added to a conical glass reaction vial, and an equal volume of 2% acetic acid in CHCl\(_3\), and 0.5 μmol MATE in 10 μl CHCl\(_3\). The coupling reaction was stopped by addition of 200 μl of 300 μM ICN, Ltd., United Kingdom) was added to 10 μl PBS in a separate vial and left agitated overnight. The beads were spun and washed three times in PBS described previously (9). Briefly, for a typical reaction using 1–10 mCi Na\(^{131}I\), 1–10 μl Na\(^{131}I\) (as above) was added to a conical glass reaction vial, and an equal volume of 2% acetic acid in CHCl\(_3\), and 0.5 μmol MATE in 10 μl CHCl\(_3\). The coupling reaction was stopped by addition of 200 μl of 300 μM aqueous glycine. Labeled antibody was purified using a Sephadex G25 column as described above.

**Immunoreactive Fraction Measurements.** One hundred μl of Sepharose beads (CNBR Sepharose, Pharmacia, Sweden) coated with 5 mg/ml of ICR55 were suspended in 1 ml of triton extract of BT474 cells rich in c-erbB2 and agitated overnight. The beads were spun and washed three times in PBS containing 1 × 10\(^{-4}\) M phenyl methyl sulphonyl fluoride, 1% Triton X-100 (Sigma Chemical Co.) and 3% FCS. Labeled antibody (3 × 10\(^{10}\) cpm) was added, and the mixture was rotated for 1 h at room temperature. The beads were spun down, and the supernatant was removed. The beads were again washed in the same solution, and the supernatant was removed. The beads and the wash were counted (Innotron Hydragramma, United Kingdom). The immunoreactive fraction was expressed as the percentage of the total counts that remained bound to the beads. Controls were performed in the absence of antigen to assay for nonspecific binding. Protein concentration was determined from \(A_{280}\) using an extinction coefficient of 1.3, measured by UV spectroscopy (Pye Unicam SPE 550).

**Mice.** In vivo experiments were performed in female nude mice ages 6–8 weeks. Mice were kept in Maximiser Laminar flow cages and given sterilized food and water ad libitum. At age 5 weeks, the mice received a general anesthetic. Two mm\(^3\) pieces of MDA MB 361 tumor were introduced into 2 flank sites through a dorsal incision (13), and 14 days later, the tumors had volumes calculated from caliper measurements of 0.21 ± 0.14 cm\(^3\) (SD). There were no significant differences in the initial sizes of tumors between the groups.

**Tissue Distribution Studies.** Mice were anesthetised using enfurane delivered by a commercial vaporizer (Halovet, United Kingdom), and labeled antibody was injected into the jugular vein in a volume of 100 μl of PBS. Mice were killed at intervals after injection, and the organs were removed, weighed, and counted (Packard Autogamma). Tissue uptake was expressed as %ID/g. For whole-body clearance studies, mice were counted at regular intervals using a large-bore well counter (Nuclear Electrics Ltd., United Kingdom).

**Radioimmunoassay Studies.** When mean tumor diameters reached 0.5–0.75 cm, mice were anesthetised, and 131\(^I\)-labeled ICR12 was administered as above. Tumors were measured at regular intervals in two perpendicular dimensions.

Volume was calculated using the formula \(V = \frac{1}{6}(\pi/6)(D1^2-D2^2D3)\), assuming the third dimension is the mean of the two measured diameters. Animals were killed when tumor diameters exceeded 1 cm, at which time tumors were excised, weighed, and fixed in methacarn for histological processing.

**Results**

**Effect of Labeling Method on Immunoreactive Fraction.** ICR12 was labeled as described above. Specific activity was calculated by measuring the activity in the fraction eluted from the PD10 column and measuring the protein concentration. The results of the effect of increasing specific activity on the immunoreactive fraction are shown in Fig. 1. The immunoreactive fraction decreased progressively from a value of 91%, with increasing specific activity up to 50 mCi/mg in the case of the antibody labeled by the IodoGen method; however, the antibody labeled by the MATE retained its immunoreactive fraction within the range studied (1–16 mCl/mg).

**Effect of Protein Dose on the Pharmacokinetics of 131\(^I\)-labeled ICR12.** Twenty-four age- and tumor size-matched nude mice bearing bilateral MDA MB 361 human breast cancer xenografts with a mean weight of 0.225 ± 0.14 g were given 100, 50, or 25 μg of 131\(^I\)-labeled ICR12 labeled by the IodoGen method in 100 μl of 0.9% NaCl. The %ID/g in target tissue was calculated on days 1, 2, 3, and 6 after injection, and the activity:gram ratio remaining in the blood and tumors was calculated. The results are shown in Fig. 2. Analysis by two-tailed Student’s \(t\) test, assuming equal variance for all data, demonstrated a highly significant reduction of the tumor:blood ratio between the mice administered 100 μg and those with 50 μg (\(P < 0.0005\)) and 25 μg (\(P < 0.0001\)) doses.

**Comparison of Whole-Body Retention of ICR12 Labeled by the IodoGen and MATE Methods.** Fourteen nude mice bearing bilateral MDA MB 361 human breast cancer xenografts were given 5 μg of 131\(^I\)-labeled ICR12 using the IodoGen method (\(n = 9\)) or by the MATE method (\(n = 5\)) at a specific activity of 1–2 mCi/mg.

Whole-body 131\(^I\) retention was measured at various time points postadministration, and the decay-corrected %ID/g was calculated. These data are shown in Fig. 3. The data were analyzed by calculating the area under the time-activity curve for each animal and testing for differences between groups using Student’s \(t\) test. These calculations
show that the area under the curve for the MATE-labeled conjugate is significantly less than that for the iodogen labeling ($P < 0.0001$).

**Comparison of in Vivo Distribution of $^{131}$I-labeled ICR12 Labeled by the IodoGen and MATE Methods.** Mice bearing bilateral MDA MB 361 tumors were given injections of 10–25 $\mu$g ICR12 labeled with $^{131}$I using the IodoGen or MATE methods. The results are summarized in Fig. 4. The %ID/g in tumors at $T = 0$ was extrapolated: (IodoGen: 24.3, 95%, 19.88–29.66; MATE: 16.28, 95%, 12.18–21.76). The data were analyzed to see if there were detectable differences in the gradients. Using the formula $t = b_1 - b_2/SE(b_1 - b_2)$ on $n_1 + n_2 - 4$ df(14) ($b =$ gradient of slope; df = degrees of freedom), the gradients were not found to differ significantly for blood ($P < 0.3$); however, the decay of residence of activity within tumors was significantly longer in the group given MATE-labeled ICR12 than Iodo-

![Fig. 3. Whole-body retention of $^{131}$I-labeled ICR12 by the IodoGen method (○) or the MATE method (□) against time in mice bearing MDA MB 361 tumors. Five $\mu$g of labeled antibody/mouse. Curves differ ($P < 0.0001$); $n = 9$, IodoGen; $n = 5$, MATE. Points, mean; bars, SD.](image)

Radioimmunotherapy of MDA MB 361 Xenografts in Mice. **nu/nu** mice bearing bilateral MDA MB 361 tumor xenografts received 50–600 $\mu$Ci $^{131}$I-labeled ICR12 using the IodoGen method (20 mCi/mg), 600 $\mu$Ci $^{131}$I-labeled ICR12 using the MATE method (20 mCi/mg), and 600 $\mu$Ci $^{131}$I-labeled ICR16 using the IodoGen method (20 mCi/mg), or PBS in a volume of 100 $\mu$l/mouse. The mean initial tumor sizes were similar in the two groups [0.35 cm$^3$ MATE versus 0.29 cm$^3$ IodoGen; $P < 0.58$ (not significant)]. Dose-dependent tumor growth inhibition was seen at all doses of $^{131}$I-labeled ICR12 by the IodoGen method above and including 100 $\mu$Ci/mouse (data not shown). Fig. 5 compares the growth curves of the tumors treated by selected $^{131}$I-labeled monoclonal antibody preparations with untreated controls. Statistically significant reduction in size was seen between controls and tumors treated with 600 $\mu$Ci $^{131}$I-labeled ICR12 by either method ($P < 0.0001$; Student's $t$ test for all data). The final sizes of tumors were significantly smaller in the IodoGen-labeled group than in the MATE-labeled group ($P < 0.0005$; Student's $t$ test for all data). The experiment was terminated before the IodoGen-labeled tumors grew to larger than their zenith size, and it was not possible to test statistically the time to reach a particular size greater than the zenith. From the graph, it may be assumed that the growth delay in the MATE group was approximately 12 days and in the IodoGen-labeled group >24 days. Six hundred $\mu$Ci $^{131}$I-labeled ICR16 had no influence on growth. ICR12 is known to have no influence on growth at doses up to 300 $\mu$g single dose or 500 $\mu$g weekly for 3 times (13).4 Histological examination of tumor sections taken at the time of termination of the experiment demonstrated that the control tumors are characterized by an absence of necrosis and little stromal tissue. The MATE-labeled group demonstrates areas of necrosis with little stromal tissue overgrowth.

The tumors from the IodoGen group show proportionately fewer viable cancer cells in the tumors, with marked pleomorphism and cytoplasmic hyperchromatism of the remaining cells often with pyknotic nuclei. These tumors are extensively composed of stromal cells.

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4 Unpublished data.

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Fig. 2. Levels of $^{131}$I-labeled ICR12 as a function of time for blood (a) and MDA MB 361 tumor xenografts (b). c, tumor:blood ratio. Injected doses of ICR12: ○ 100 $\mu$g, □ 50 $\mu$g, △ 25 $\mu$g. Specific activity, 1 $\mu$Ci/$\mu$g $^{131}$I-labeled ICR12. Student's $t$ test for all data in c: $P < 0.0001$ between 100 versus 25 $\mu$g; $P < 0.0005$ between 50 versus 25 $\mu$g. a, 2/point. b and a, 4/point. Points, mean; bars, SD.
Discussion

Radioimmunotherapy has been shown in a number of preclinical studies to be a promising treatment but has had limited success in clinical trials of solid tumors in patients. There are a number of explanations for this; however, the major factor has been the unacceptable irradiation of nontumorous tissues, predominantly the bone marrow. As predicted by the MIRD dosimetric calculations (17), the relative exposure of tumor and bone marrow to the radioactivity administered is clearly critical in determining the effectiveness of the treatment. This is itself a function of the initial quantity of monoclonal antibody that localizes within the tumor and the rate of clearance of the antibody from the tumor and blood. We have demonstrated that the antibody dose is critical to the initial tumor:blood ratio, and that the less monoclonal antibody that is given, the greater the localization ratio. This can be explained by the fact that the c-erbB2 p185 receptor is relatively scarce (9 X 10^6 receptors/cell; Ref. 13), and that the binding site for ICR12 on the external protein epitope becomes saturated. Higher doses of monoclonal antibody than that required to saturate the receptors will lead to reduced levels of tumor relative to blood and, therefore, to bone marrow.

The limitation of protein dose makes more critical the specific activity of the radiolabeled antibody. We have demonstrated that the immunoreactive fraction of the ICR12 falls as the specific activity increases, presumably due to the increased likelihood of radioiodine at the binding site of the antibody molecule. For a given dose of radioactivity, there is, therefore, a trade-off between low specific activity to preserve immunoreactive fraction and a low protein dose to preserve tumor localization. Alternative radiolabeling methods offer a number of potential advantages.

The MATE method has been shown previously to increase the tumor:blood ratio for a F(ab')2 fragment (8); however, we were not able to show this for intact ICR12. A likely explanation for this is that ICR12 is less susceptible to impairment of function due to electrophilic substitution of ^131I using the IodoGen method, compared with reported studies using other antibodies. MATE-labeled monoclonal antibody has been shown previously to have prolonged retention within tumors, resulting in increased tumor irradiation, and we have demonstrated that this is the case in our system. Finally, it is postulated that the MATE labeling method may increase the specific activity that can be achieved without loss of immunoreactive fraction, allowing a desirable reduction in the protein dose that needs to be administered. Because of the requirement for high protein concentration (~3 mg/ml) in the antibody coupling reaction using MATE, it was impractical to investigate MATE labeling at specific activities above 16 mCi/mg; however, the results indicate that it is possible to achieve higher specific activities with MATE labeling than with IodoGen labeling without loss of immunoreactive fraction. In a previous study using an analogous iodination agent, N-succinimidyl 3-[^131I]iodobenzoate, antibody labeling with retention of immunoreactive fraction was performed at specific activities up to 26 mCi/mg (18). We are able to conclude from these data that the MATE method gives immunoreactive fractions of the ICR12 that are at least as good as those found with the IodoGen technique and probably better. Our
results indicate that $^{131}$I-labeled ICR12 administered i.v. is effective in temporarily arresting the growth of MDA MB 361 tumors, and that histological analysis of the tumors treated by this technique shows that there has been extensive tumor cell death. Our results show that the IodoGen labeling technique gave longer tumor growth inhibition than the MATE technique at the same administered activity, despite the dosimetric estimates predicting that the MATE technique might give better results.

The MATE-labeled conjugate appears not to localize as well within the tumors as IodoGen-labeled ICR12, and the dosimetric variable that causes the predicted dose to be greater is the prolongation of the tumor residence of the activity. As we have discussed, the 2-fold differences in rate of clearance of activity from the tumors between the groups are statistically significant. It should be stressed that it is the clearance of activity and not antibody from the tumor that determines the efficacy of treatment. The lack of therapeutic advantage for MATE in light of observations of higher immunoreactive fraction and tumor residence is perplexing. Although the differences in tumor size between the IodoGen and MATE groups was small (0.35 cm$^3$ MATE; 0.29 cm$^3$ IodoGen), it is possible that this could contribute to the differences in therapeutic efficacy between the two groups. Assuming spherical tumor geometry, these volumes correspond to tumors with radii of 4.4 and 4.1 mm for the MATE and IodoGen groups, respectively. Because the 90th centile distance for $^{131}$I $\beta$ particles is 0.82 mm (19), it is possible that limited penetration of the antibody within the tumor could spare cells in the interior of larger tumors but not smaller tumors, facilitating irradiation in the IodoGen group. In addition, because of the reduction in the whole-body retention of MATE-labeled activity, the dose to the tumors from outside the tumors is less, which may contribute to the worse than predicted results using MATE-labeled ICR12 for radioimmunotherapy. These encouraging results suggest that radioimmunotherapy may have a role in the adjuvant treatment of breast cancer, and that further development of the technique is warranted. Alternative methods of radioiodination of antibodies may well increase the specific activity of antibody that can be achieved without loss of immunoreactive fraction, which will mean that a reduced dose of monoclonal antibody can be given. The MATE method appears to be advantageous in this respect, although for ICR12 the advantages of a potentially increased immunoreactive fraction are not sufficient to make the additional complexity of the MATE labeling method worthwhile.

Other antibodies that are more liable to lose immunoreactivity as a result of labeling by the IodoGen method may benefit more from indirect labeling by the MATE method. It is unlikely that sufficient doses of radioimmunotherapy can be given to patients with breast cancer to effectively control disseminated disease without unacceptable toxicity to nontumorous organs; however, there may be an important role in combined external beam/parenteral radiotherapy. Further methods to increase the tumor-blood activity ratio may also be important, and we are investigating two- and three-stage targeting with blood clearance to improve this ratio.

References

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