Effect of Multiple, Repeated Doses of Radioimmunotherapy on Target Antigen Expression (Breast MUC-1 Mucin) in Breast Carcinomas

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ABSTRACT

The effect of radioimmunotherapy (RIT) on target antigen expression was studied in breast carcinomas transplanted in immunodeficient mice. In nine separate experiments, a single dose of 1500 μCi of 131I-labeled monoclonal antibody (MAb) Mc5 was given to groups of mice carrying well-established, vascularized, transplantable breast tumors (MX-1). Mc5 recognizes an epitope on the tandem repeat of the breast epithelial MUC-1 mucin. This dose suppressed tumor growth for at least 20 days, after which the tumors began to regrow. At various times thereafter, tumors were removed and analyzed for target antigen expression by flow cytometry and immunohistochemistry. In no case was there any significant decrease in antigen content/cell in the tumors of treated mice compared to tumors in control untreated mice. Similar results were obtained with four other breast carcinomas (MCF-7, MDA-MB-331, MDA-MB-435, and MX-2A). To assess the effect of repeated RIT doses on target antigen expression, groups of mice with MX-1 tumors were given 2, 3, and 4 consecutive doses of 1200 μCi of 131I-labeled Mc5. One mouse each at 2, 3, and 4 doses (3 of 18) was cured of its tumor. Control mice were sacrificed after 50 days due to the excessive size of their tumors. Tumors from four mice from each group (2, 3, and 4 doses), after they began to regrow, were excised and analyzed for mucin content and compared to tumors from untreated mice with similar-size tumors transplanted at later dates. In none of the treated groups was there any decrease in mucin content. These results demonstrate that RIT with an anti-breast mucin MAb does not result in the appearance of antigen-negative tumor cells, thus indicating that repeated fractionated doses, which will most likely be necessary for an eventual cure of breast cancer with MAb therapy, are possible.

INTRODUCTION

MAbs3 could provide an alternative to conventional adjuvant therapy of metastatic breast cancer by selectively targeting tumors. In experimental model systems, both native unconjugated and radioconjugated MAbs have proved effective in the treatment of breast carcinomas (1, 2). Radioiodinated MAbs proved to be more effective than unconjugated MAbs, being able to inhibit the growth of well-established, vascularized, transplantable human breast tumors with a single dose (1, 3). Cures of similar human breast tumors could be obtained with 90Y-labeled anti-breast mucin (MUC-1) MAbs (3—5) and with the concomitant use of radiosensitizers (6). Clinical demonstration of the breast tumor-selective binding of anti-mucin MAbs was provided in clinical trials in which an 111In-labeled anti-CEA, selection of CEA-negative cells was observed (13); however, a resurgence of antigen expression occurred after further growth of the tumors (13). The superior results of RIT with MAb B1 of B-cell lymphomas in humans (14) have been attributed to the lack of modulation of its antigen (CD20), compared to the CD19 and CD22 lymphoma antigens that are modulated, either being internalized or shed (15, 16). Because of the apparent need for repeated doses in the treatment of solid tumors by RIT, we undertook the present study of the effect of 131I-labeled Mc5 therapy on its target antigen (breast mucin) levels in regrown transplanted breast tumors in immunodeficient nude mice after treatment with single and multiple doses.

MATERIALS AND METHODS

MAbs. MAb Mc5 was originally prepared by immunizing mice with de-lipidated human milk fat globule and by selection of hybridomas secreting MAbs that bound to the human milk fat globule membranes and breast carcinoma cell lines but not to the membranes of non-breast cell lines, as described previously (17, 18). Mc5 recognizes an epitope on the tandem repeat region of the high molecular weight, highly glycosylated, breast MUC-1 mucin (17—19).

Experimental RIT. Immunodeficient nude mice (nu/nu) with a BALB/c background were purchased from Life Sciences, Inc. (St. Petersburg, FL). Female BALB/c-nu/nu mice were grafted with transplantable human breast carcinomas at 7—8 weeks of age (body weight, 19—24 g). Grafts (approximately 20 mm3) were sterilized implanted through a flank incision midway between the front and hind legs and then gently pushed above the hip, and the wound was closed with metal clips. The grafted tumors were allowed to grow for about 2 weeks, to the size of approximately 100 mm3, before the first dose of 131I-labeled Mc5 was given i.p. The protocol was approved by the Institutional Animal Care and Use Committee. If the tumors became ulcerated or if the animals seemed to be uncomfortable or suffering, the animals were sacrificed following IACUC-approved euthanasia procedures. Mc5 was purified by protein-A affinity chromatography from protein-free culture medium. Iodinations were carried out with Na131I (specific activity, 8—12 Ci/mg; DuPont New England Nuclear Research Products, Boston, MA) using chloramine T (0.5 mg/ml; Ref. 20) at a MAb concentration of 4—10 mg/ml. Mc5 was labeled to a specific activity of 9—12 mCi/mg protein, which represented approximately 1 iodine molecule/antibody molecule. With this radiolabeling protocol, an immunoreactivity of 50—75% is routinely obtained as measured by binding to antigen-coated beads. The labeled antibody is separated from free iodine by passing the labeled material through a Sephadex G-25 column. Fifteen hundred μCi were given in the single-dose experiments, and 1200 μCi were given for each dose in the multiple-dose experiment.
Tumors were transplanted on day 20, and a single dose (1500 @Ci) of radiolabeled MAb was given on day 0. The growth curves are plotted on semilog scale to allow a better comparison of growth curves of the tumors in the treated and untreated groups. There were four animals/group. Points, mean tumor volume ± S.E.

Tumor volumes were assessed by measuring width, length, and height, multiplying the dimensions together, and dividing the product by 2, as described previously (1, 2). The percentage of inhibition of growth was calculated, in which the growth of the treated tumor was compared to the growth of the tumors in the untreated control animals. The transplantable breast carcinomas used in these studies include three estrogen receptor-negative tumors, MX-1 (1), MX-2A (both obtained from Dr. Bogden, EG and G Mason Research Institute, Worcester, MA), and MDA-MB-435 (21), and two estrogen receptor-positive tumors, MCF-7 and MDA-MB-331 (21). The latter three transplantable tumors we established from cell lines (21); MDA-MB-331 and MDA-MB-435 were obtained from Dr. Relda Cailloue, and MCF-7 was obtained from the American Type Culture Collection. The estrogen receptor-positive tumors are estrogen-dependent for growth because estrogen pellets implanted under the skin were required for growth in nude mice (2).

Flow Cytometry Analysis of Surface and Total Mucin Content. Mucin content/cell was quantitated by flow cytometry as described previously (22). For this purpose, the tumors were excised and sliced into small pieces with a scalpel. Then they were suspended in 0.02% EDTA in Ca²⁺/Mg²⁺-free isotonic buffered solution, drawn through a 16-gauge needle several times, and were let stand for 30 min. The tubes with cells were inverted to resuspend cells, the tissue chunks were allowed to settle, and the suspended cells were decanted. All of the above procedures were done at room temperature. The suspended cells were centrifuged and then aliquoted into two fractions, one of which was fixed with cold 70% ethanol in PBS for 30 min at 4°C for detection of total mucin/cell, and the other was stored unfixed at 4°C for detection of surface mucin content. The cells were then incubated with the FITC-conjugated MAb Mc5 dissolved in PBS containing 1% calf serum for 30 min at 4°C. MAb were conjugated with FITC by the method of Kearney and Lawton (23). A separate aliquot of fixed and unfixed cells was incubated with a nonspecific FITC-conjugated IgG1 (Coulter Immunology, Hialeah, FL). The stained cells were rinsed once with 2 ml of PBS and 1% calf serum, resuspended in the same buffer, filtered through a 35-µm Nitex filter, and analyzed on a Coulter EPICS 753 flow cytometer. The percentage of positive cells and mean channel number (fluorescent intensity) for the positive cells were determined using the Easy 88 Immuno program. Quantitation of relative staining intensity was done using Simply Cellular beads (Flow Cytometry Standards Corp., Research Triangle Park, NC, Ref. 24). During each run, the Simply Cellular beads were stained in an identical fashion as the cells and run on the flow cytometer under the same conditions (power, high voltage, and gain), and from the mean channel number of the beads, the relative mean channel number of the cells was then standardized for comparison of the staining intensity of cells run at different times. All mean channel numbers were then normalized to a given gain and high voltage. Even though we normalized all values, comparison for the effect of treatment on breast mucin content was done with tumors from treated and untreated mice analyzed on the same day. In the single-dose experiments, tumors were transplanted at the same time, using portions of the same tumor for both treated and untreated animals.

Immunohistochemistry. For immunohistochemical staining of tumor tissues, pieces of the same tumors prepared for flow cytometry were fixed with Bouin’s fixative, embedded in paraffin, and sectioned. Five-µm sections were deparaffinized and stained with Mc5 (5 µg/ml) using an immunoperoxidase technique as described previously (25).

RESULTS

The purpose of these studies was to examine the effect of 131I-labeled MAb therapy of human breast carcinomas transplanted into immunodeficient nude mice on the expression of the target antigen (MUC-1 breast mucin). In the initial experiments, immunodeficient nude mice were grafted with transplantable fragments of the human breast carcinoma MX-1. Approximately 2–3 weeks after transplantation, when the tumors were well vascularized and about 100 mm³ in volume, a group of mice were treated with a single dose of 1500 µCi of 131I-labeled Mc5 (Fig. 1). Such treatment typically results in the arrest of tumor growth and often results in an initial decrease in tumor volume (1; Fig. 1). Compared to control untreated mice, the percentage of inhibition of growth at 26 days after treatment averages 96%, based on tumor volumes (1). However, with this dose, the tumors usually begin to regrow at about 20 days after treatment. An example of such an experiment is presented in Fig. 1, in which the differences between the tumor volumes of treated and untreated tumors are significantly different after the first few days after treatment. We have previously shown that 131I-labeled nonspecific mouse IgG does not inhibit tumor growth (1).

At various times after treatment (40–126 days), 2 treated and 2 untreated animals were sacrificed, and their tumors were excised and analyzed for mucin by flow cytometry using MAb Mc5 (Fig. 2). Both surface and total mucin content were determined, as well as the percentage of positive cells (Table 1). For each experiment, the animals received transplants at the same time with pieces of the same transplanted tumor, the treatment was a single dose of radiolabeled MAb given at the same time, and the flow cytometry analysis was done on the same day for each experiment to reduce variation due to tumor heterogeneity and transplantability and to reduce differences in the instrument setup, laser power, fluorescently labeled MAb, and staining procedures. In all cases, tumors in the untreated animals were con-
These tumors differed from MX-1 in total antigen content, percentage of positive cells, and intracellular distribution (surface versus cytoplasmic expression). Also, whereas MX-1, MX-2A, and MDA-MB-331 of the five tumors tested (Table 1). For the MX-2A tumor, there was also no decrease in the intensity of either surface or total staining with MAb Mc5 for four (MX-1, MDA-MB-331, MDA-MB-435, and MDA-MB-331 are estrogen and progesterone receptor-positive (21)). In addition, they differed in growth rates (21). Flow cytometry analysis of the treated tumors compared to the untreated controls showed no significant decrease in the percentage of positive cells, relative surface/cytoplasmic staining, or the intensity of surface staining and total staining with MAb Mc5 for four (MX-1, MDA-MB-331, MDA-MB-435, and MCF-7) of the five tumors tested (Table 1). For the MX-2A tumor, there was also no decrease in the intensity of either surface or total staining; however, there seemed to be a decrease in the percentage of positive cells and a shift to more surface staining. The increase in antigen-negative cells in the treated tumors could be due to an increase in normal cells infiltrating the tumor as a result of the treatment. Also, because the percentage of positive cells was low to begin with in the MX-2A tumor, only a small change in the number of negative cells would have a large effect on the percentage, which may not be significant.

These results show that a single dose of RIT does not result in the appearance of target antigen-negative cells; however, it is still possible that multiple doses could do so. Therefore, nude mice were transplanted with the MX-1 tumor, and groups (6 mice each) were treated with 2, 3, and 4 doses of 1200 mCi of 131I-labeled Mc5. This dose, which is below the MTD for a single injection, was selected to reduce toxicity because of the multiple doses and to favor selection of antigen-negative cells. As seen in Fig. 3, the multiple treatment suppressed tumor growth and allowed the majority of animals to survive for at least 93 days after treatment, when the experiment was terminated. Only 2 of 18 mice died during the experiment, 1 after 3 doses and 1 after 4 doses, whereas 3 of 18 mice were cured, 1 in each dosage group. The control untreated animals, which received transplants at the same time as the treated animals, had to be sacrificed by day 50 because of the size their tumors, which were, on average, 40 times larger than those in the treated animals.

To assess the effect of multiple doses on target antigen expression, tumors that had regrown after two, three, and four doses were excised (four per group) and analyzed for breast mucin content by both flow cytometry and immunohistochemical staining using MAb Mc5. The control untreated tumors were from mice that received transplants at later dates and selected for comparable sizes (Fig. 3). As shown in Fig. 3, flow cytometry analysis of mucin content with Mc5 showed that there was no decrease in the antigen content/cell as a result of the treatment in any of the dosage groups. These results were confirmed by immunohistochemical staining (Fig. 4).

### Table 1 The effect of RIT on target antigen content in different breast carcinomas transplanted into nude mice analyzed by flow cytometry

<table>
<thead>
<tr>
<th>Tumor (mouse #)</th>
<th>% Pos. a</th>
<th>% Surface b</th>
<th>Surface intensity c</th>
<th>Total intensity c</th>
<th>Antigen index d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX-1 Untreated (18)</td>
<td>49</td>
<td>33 ± 7</td>
<td>148 ± 16</td>
<td>124 ± 14</td>
<td>60</td>
</tr>
<tr>
<td>MX-1 Treated (18)</td>
<td>51</td>
<td>34 ± 6</td>
<td>119 ± 8</td>
<td>159 ± 14</td>
<td>81</td>
</tr>
<tr>
<td>MX-2A Untreated (#2435)</td>
<td>3</td>
<td>3</td>
<td>327</td>
<td>464</td>
<td>14</td>
</tr>
<tr>
<td>MX-2A Treated (#2444)</td>
<td>0.09</td>
<td>67</td>
<td>383</td>
<td>636</td>
<td>0.6</td>
</tr>
<tr>
<td>MDA-MB-435 Untreated (#2727)</td>
<td>27</td>
<td>&lt;0.01</td>
<td>366</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>MDA-MB-435 Untreated (#2730)</td>
<td>20</td>
<td>&lt;0.01</td>
<td>151</td>
<td>69</td>
<td>14</td>
</tr>
<tr>
<td>MDA-MB-435 Treated (#5001)</td>
<td>34</td>
<td>0.03</td>
<td>256</td>
<td>81</td>
<td>27</td>
</tr>
<tr>
<td>MDA-MB-435 Treated (#2737)</td>
<td>41</td>
<td>&lt;0.01</td>
<td>295</td>
<td>69</td>
<td>28</td>
</tr>
<tr>
<td>MCF-7 Untreated (#774)</td>
<td>51</td>
<td>49</td>
<td>157</td>
<td>146</td>
<td>74</td>
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<tr>
<td>MCF-7 Treated (#773)</td>
<td>60</td>
<td>52</td>
<td>150</td>
<td>208</td>
<td>124</td>
</tr>
<tr>
<td>MDA-MB-331 Untreated (#463)</td>
<td>61</td>
<td>79</td>
<td>294</td>
<td>251</td>
<td>153</td>
</tr>
<tr>
<td>MDA-MB-331 Treated (#2751)</td>
<td>87</td>
<td>90</td>
<td>364</td>
<td>283</td>
<td>245</td>
</tr>
</tbody>
</table>

a % Pos. percentage of positive cells.
b % Surface, percentage of positive cells that had surface staining.
c The intensity of surface staining and total staining (surface and cytoplasmic) are expressed as the mean relative channel number determined by flow cytometry, as described in "Materials and Methods."
d Antigen index is the product of the percentage of positive cells and the total antigen content (mean relative channel number).

d Number of tumors. For MX-1 tumors, the means of the individual values presented in Fig. 2 are given.

Fig. 3. Effect of multiple fractionated doses of 131I-labeled MAb Mc5 on the growth of MX-1 breast carcinomas in immunodeficient mice. Tumors were transplanted on day 0, 22, 47, and 72, respectively (indicated by arrows under the abscissa). Four animals were sacrificed on day 47 for 2 doses (A), day 67 for 3 doses (B), and day 85 for 4 doses (C), and tumors were analyzed for mucin content by flow cytometry and immunohistochemical staining. Box insert, flow cytometry analysis of relative mucin content (mean relative channel number ± S.E.M.) of tumors after one, two, three, and four consecutive doses (four mice/group) and tumors from control untreated mice. The values for one dose are from the treated and untreated MX-1 tumors presented in Table 1.

Fig. 2 presents the relative mucin contents (mean relative channel number) of tumors from treated and untreated animals for each experiment. The important conclusion from these experiments is that in none of the experiments was there any decrease in mucin content as a result of the treatment.

To determine whether this lack of selection of antigen-negative tumor cells by RIT is particular to the MX-1 breast tumor, similar experiments were done with four other breast carcinomas (Table 1). These tumors differed from MX-1 in total antigen content, percentage of positive cells, and intracellular distribution (surface versus cytoplasmic expression). Also, whereas MX-1, MX-2A, and MDA-MB-435 are estrogen and progesterone receptor-negative, MCF-7 and MDA-MB-331 are estrogen and progesterone receptor-positive (21). In addition, they differed in growth rates (21). Flow cytometry analysis of the treated tumors compared to the untreated controls showed no significant decrease in the percentage of positive cells, relative surface/cytoplasmic staining, or the intensity of surface staining and total staining with MAb Mc5 for four (MX-1, MDA-MB-331, MDA-MB-435, and MCF-7) of the five tumors tested (Table 1). For the MX-2A tumor, there was also no decrease in the intensity of either surface or total staining; however, there seemed to be a decrease in the percentage of positive cells and a shift to more surface staining. The increase in antigen-negative cells in the treated tumors could be due to an increase in normal cells infiltrating the tumor as a result of the treatment. Also, because the percentage of positive cells was low to begin with in the MX-2A tumor, only a small change in the number of negative cells would have a large effect on the percentage, which may not be significant.
DISCUSSION

The present results demonstrate that with breast MUC-1 mucin as a target for RIT of breast tumors in nude mice, a single dose that significantly inhibits tumor growth does not select for antigen-negative cells in tumors that regrow. We obtained these results repeatedly with the MX-1 tumor, which is highly susceptible to this treatment. Also, four other transplantable breast carcinomas gave similar results. Moreover, repeated doses (up to four) extended the therapeutic effect, but without any reduction of target antigen content in the tumors that regrew. A small percentage of the mice were cured of their tumors after multiple doses. These results demonstrate that the emergence of mucin-negative tumor cells does not occur as a result of RIT using this breast surface antigen as a target.

Different results were obtained when native unconjugated MAbs were used in experimental therapy of breast cancer, in which selection of antigen-negative cells did occur (2). In a recent report in which human colon carcinomas transplanted into nude mice were treated with a 90Y-conjugated MAb against CEA, there was a selection of tumor cells that had a significant reduction in CEA content (26). However, in subsequent studies, there was a resurgence of CEA-positive cells at about 6 months after additional transplantation of tumors from the treated mice (27).

The difference between the present results and those from therapy with unconjugated MAbs (2) could be due to the mode of treatment. In the case of treatment of breast tumors in nude mice with a mixture of unconjugated MAbs (2), administration of the MAbs was begun at the time of injection and repeated every other day over a long period of time (2), whereas in this study, the tumors were well established.

Fig. 4. Immunoperoxidase staining with MAb Mc5 of transplanted MX-1 breast tumors in immunodeficient mice after one, two, three, and four doses of 131I-labeled Mc5 and an untreated control tumor. Secondary antibody (2° Ab) alone gave no staining.
radiolabeled MAbs were given, and the repeated doses were given at least 2-week intervals. The unconjugated MAbs must interact with each of the tumor cells individually to be effective, so antigen-negative cells would not be affected and would be expected to have a selective advantage. With radiolabeled MAbs, each tumor need not bind the MAb, and binding to antigen surrounding the tumor can still provide a lethal dose of radioactivity. Also, cells with larger amounts of antigen are not necessarily killed selectively because the radiation would be expected to preferentially kill cells in S-phase.

The difference between these results in treating breast cancer with radiolabeled MAbs against breast mucin and those of studies of treatment of colon carcinoma with $^{131}$I-labeled anti-CEA in nude mice could be due to at least two factors: (a) differences in the way the tumors were transplanted into the nude mice; and (b) the difference in the target antigen. The colon carcinoma cells were injected i.p. as a single-cell suspension that resulted in multiple micrometastases in the peritoneal cavity, whereas the breast carcinomas were transplanted s.c. as a single tumor piece that resulted in a single solid tumor. The size of the tumors may thus contribute to the difference in results because in the colon carcinoma, the treatment was done when the micrometastases were about 1 mm$^3$ in size, whereas with the breast carcinomas, the mice were treated when the single tumors in each mouse were about 100 mm$^3$. With the breast tumors, even if a relatively same percentage of cells are antigen-positive, their presence in a single tumor may still provide enough target antigen to have a therapeutic effect, but not selectively for only antigen-positive cells due to the proximity of the positive and negative cells. With the colon carcinoma, the multiple micrometastases resulting from injection of a cell suspension would allow spatial separation of the antigen-positive and antigen-negative micrometastases, in which some of the negative micrometastases would receive sublethal doses of radiation. Unfortunately, the authors did not indicate how many micrometastases were examined immunohistologically, so it is not possible to evaluate how general their result is (26). The fact that there is a resurgence of CEA-positive tumors after subsequent transplantation (27) indicates that the loss of antigen is only transitory.

Another factor that may be responsible for the difference in the tumor cell selection as a result of RIT in the two systems could be the different nature of the two target antigens. Perhaps breast tumors require expression of breast mucin for their survival, whereas expression of CEA may not be essential for the survival of breast cells. CEA is characteristic of the colon and is often expressed at high levels in colon carcinomas (28). It is also produced by some breast carcinomas (29). The expression of CEA by breast carcinomas is thought to be due to deregulation associated with malignancy, as is the case for other oncofetal antigens (30). Breast mucin is detectable in virtually all breast tumor cells at variable levels, depending on the MAb used to detect it (17). Also, breast mucin is polymorphic at both the DNA and mRNA levels (31), is multipotent due to the presence of amino acid tandem repeats with multiple immunodominant regions (32), and exhibits a high degree of epitopic heterogeneity in which at least some epitopes are always expressed (17). The function of the breast mucin is not known, but it has been suggested to act as a lubricant at the epithelial cell surface, as is characteristic of mucins, and may contain specific cell receptors (33).

Clinical trials for treatment of B-cell lymphoma with radioiodinated anti-CD20 MAb B1 also support our conclusion that RIT targeted to appropriate antigens does not result in the appearance of antigen-negative tumor cells (14). Durable remissions have been achieved with this antibody in chemotherapy-refractory patients, and CD20-positive cells reappear in the patients after 2—3 months (14).

In previous experimental RITs of transplantable human breast tumors with $^{131}$I-labeled MAbs (1, 5, 6), there was no total eradication of the tumors. Nevertheless, in the present experiments, 3 of 18 mice treated with repeated doses were cured of their tumors. This could indicate that repeated sub-MTDs could yield cures in the clinical setting. This approach could be tempered by the response to heterologous immunoglobulin usually found after the injection of murine MAb radioconjugates.

The significance of these results is that when breast mucin is used as a target for RIT, repeated doses can be given without selection of antigen-negative cells. It is expected from both preclinical (1, 2) and clinical trials (34) that more effective RIT of breast cancer will be obtained with multiple repeated doses. In the treatment of breast cancer patients with mouse MAbs, only a single dose is possible because of the development of human anti-mouse antibodies (7).

Recently, we have developed both chimeric (35) and humanized (36) MAbs against human breast tumors, which are immunogenic or nonimmunogenic and allow repeated doses.

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