ABSTRACT

Breast tumors are susceptible to attack by unconjugated anti-human milk fat globule monoclonal antibodies (MoAbs) and most particularly by their mixture (cocktail) (Cancer Res., 47: 532–540, 1987). In the present study the same MoAbs (Mc1, Mc3, Mc5, and Mc8) labeled with 131I, either singly or in cocktails, were used for a similar purpose. Biodistribution studies showed that a transplantable human breast tumor line (MX-1) implanted in BALB/c nude mice (nu/nu) had the maximum incorporation of injected 131I-MoAbs at day 4 while levels in circulation and in normal tissue declined steadily from day 1. Also, these studies showed that the amount of radiolabeled Mc3 MoAb incorporated by MX-1 tumors was greater than that for cocktail of MoAbs and MoAb Mc5.

Tumor destruction by injected 131I-MoAb cocktail was shown in therapy experiments to be dose dependent. A single injection (500 µCi/mouse) of 131I-MoAb Mc3, or of cocktail, produced large breast tumor volume diminution and inhibition of growth for up to 30 days while a similar dose of 131I-labeled control IgG had no effect. A second dose of 500 µCi 131I-MoAb of Mc3 or of cocktail, injected at an appropriate interval, again diminished tumor mass significantly and inhibited its growth for another 20 days. In control experiments, non-breast tumors (colon) were marginally affected by the 131I-MoAbs. These results show that the systemic injection of radioiodinated MoAbs against human milk fat globule destroy the epithelial cells of human breast tumors and control their growth for an appreciable length of time. Radioiodinated MoAbs proved to be more effective than unconjugated MoAbs in reducing breast tumor mass and also in inhibiting growth for longer periods of time at immunoglobulin doses 100 to 200 times lower. Further exploration of their role in breast cancer treatment seems warranted by these results.

INTRODUCTION

Breast epithelial cell antigens can comprise different systems. Among them are: class I, normal antigens shared with cells of most other tissues (1); class II, antigens shared with cells of a few other tissues but with a preponderance in the breast (2) that could be referred to as characteristic of the breast; class III, antigens present only in the gland itself being truly breast tissue specific such as casein and α-lactalbumin; class IV, breast tumor antigens shared with other tumors of the organism (3); and finally, class V, specific breast epithelial tumor antigens.

Breast tissue specific markers, such as casein and α-lactalbumin, are hormonally regulated, and with the advent of hormone independence in tumors their levels become negligible (4). Using HMFG2 membranes as immunogens, we originally produced polyclonal antibodies against the breast characteristic antigens (2) and now have created MoAbs. MoAbs against high, intermediate, and lower molecular weight antigens (5, 6) were prepared. These MoAbs bound antigens that could be classified as class II above and also bound with high prevalence in breast tumors as shown by immunohistopathology (7, 8). Simultaneous with our initial discovery that breast epithelial antigens detected by polyclonal antibodies are present in the circulation of breast cancer patients (9), lower molecular weight antigen(s) were also detected in their blood by MoAb Mc3 (9); thus, strong consideration is given to the possible interference of circulating antigens in MoAb therapy. In spite of these high circulating antigen levels 131I-labeled antibodies were useful in imaging studies of breast tumors (10, 11).

At the clinical level, unconjugated (12–14) and conjugated MoAbs (15–17) have been used previously by researchers to attack hematological and other malignancies. In the specific case of melanoma (18), immunoconjugates of ricin A chain to an anti-melanoma antibody are claimed to have had success in the treatment of at least 30% of the melanoma patients in a recent clinical trial (18). In our hands one of these MoAbs against breast epithelium (Mc5 directed to the Mr 400,000 mucin) was successful in destroying breast tumor cells in monolayers when conjugated to diphtheria toxin A chain (19).

Unconjugated, anti-breast epithelial MoAbs have been used successfully to attack experimentally human breast tumors in vivo (20–22). In a detailed study using four MoAbs (Mc1, Mc3, Mc5, and Mc8) that we originally created against the HMFG, we explored their use extensively in unconjugated form by experimentally treating, both at the time of transplantation and also after their establishment, human breast tumors in nude mice (23, 24). These MoAbs showed independent ability to arrest the growth of breast tumors, and their action could be heightened by their use as a mixture or cocktail (23, 24). Heterogeneity of antigenic expression of breast tumor cells (25, 26) could have precluded the complete eradication in most cases of all the tumor cells by unconjugated antibody treatment in our experiments (24). As an alternative, we propose the use of radioiodinated MoAbs that can destroy not only the cell to which the MoAb binds but also surrounding cells in a radius that will be directly related to the energy of the particle. Support for this approach is given by the relative empirical success of intracavitary administration (27) of a radioiodoconjugate of MoAb (3.14.A3) we had prepared (5) [presently addressed as MoAb Mc1 in our studies (6, 24)]. In this paper we describe the effectiveness of radioiodinated MoAbs against breast epithelium both singly or in mixtures in treating human breast tumors carried by nude mice.

MATERIALS AND METHODS

After tagging for identification BALB/c-nu/nu mice were used for tumor transplantation. They were obtained from Life Sciences (St. Petersburg, FL) and fed Purina mouse chow 5058 and sterilized water acidified to pH 2.5, while kept in isolation in sterile cages and bedding. After radioconjugate injection each mouse was placed in a separate cage and the bedding changed more frequently.

The transplantable human tumors, MX-1, a human breast carcinoma derived from a primary breast tumor, and CX-1, a human colon carcinoma derived from the human colon carcinoma cell line HT-29, were obtained from the EG&G Mason Research Institute, Worcester,
MA. Nude mice were transplanted through a flank incision midway between the front and hind legs with 2-3-mm³ pieces of each tumor which were pushed just anterior to the hips as already described (24). To obtain volumes for the transplanted tumors, width, length, and height were measured; the three dimensions were then multiplied and the result divided by 2; the volume obtained corresponded closely to those derived by using calculations for the volume of an ellipsoid (24). Percentage of inhibition of growth was calculated as

\[
\%IG = \left(1 - \frac{Tn}{To}\right) \times 100
\]

where To and Tn are the mean tumor volumes at day 0 (day of initial radioconjugate injection) and day n for the treated groups, respectively, and Co and Cn are the mean tumor volumes at day 0 (day of initial radioconjugate injection) and day n for the control groups respectively (28).

For biodistribution and immunotherapy experiments i.p. injections of the labeled MoAbs were administered to nude mice carrying tumors, and the animals were then placed in separate cages, fed, and provided acidified water as usual. Two mg of potassium iodide in phosphate buffered saline were injected i.p. 1 h before every injection of radioiodinated MoAb to block thyroid radioidine uptake. For tumor and organ uptake measurements the mice were sacrificed by cervical dislocation, the tumors and organs dissected and weighed, and their radioactivity was counted. In these biodistribution studies the total dose present per g of tumor or normal tissue was obtained after correcting for decay of the 131I.

Hybridoma cell lines producing MoAbs Mc1, Mc3, Mc5, and Mc8 (5, 6) or the X63B myeloma parent cell line used to produce ascites were injected into 2.6,10.14-tetramethylpentadecane primed BALB/c mice. Normal mouse serum was obtained from BALB/c animals of either sex.

Ascites was collected sterilely and the cells removed by centrifugation at 150 x g for 5 min. The ascites supernatant was then again centrifuged at 20,000 x g for 10 min at 4°C and frozen at -80°C until used. As needed the ascites supernatant was thawed, centrifuged at 40,000 x g for 15 min, diluted 1:1 with 0.05 M phosphate buffer, and filtered through a 0.2-μm cellulose acetate filter (Nalgene; Sybron, Rochester, NY) before immunoglobulin separation through a hydroxyapatite preparatory column (1.5 x 5.0 cm; Bio-Rad, Richmond, CA) with a sodium phosphate gradient (0.01 to 0.3 M) containing 0.01 mM CaCl₂. The immunoglobulin fraction from normal mouse serum was prepared in the same manner except that in addition a 40% ammonium sulfate precipitation followed by dialysis versus phosphate 0.02 M buffer, pH 6.5, was used after the 20,000 x g centrifugation.

The purified fractions of immunoglobulin were concentrated with a CX-30 Immersible (Millipore, Bedford, MA) and filtered for sterility through a 0.2-μm cellulose acetate filter (Millipore).

Pyrogen testing for endotoxin was performed (after final MoAb and normal immunoglobulin purification) using the Limulus amoebocyte lysate test (Malinckrodt, St. Louis, MO). Samples were considered pyrogen free if the amount of endotoxin was below that detectable by the test (0.06 ng/ml). All solutions were prepared in pyrogen free water.

Iodinations were carried out with 131I (8-12 Ci/mg; NEN Research Products, Boston, MA), and when required with 125I (14-15 Ci/mg; Amersham, Arlington Heights, IL). MoAbs were iodinated using chloramine-T (0.5 mg/ml) (29) and at MoAb concentrations of 4-10 mg/ml. Samples were counted in a gamma counter (Multi-Priaz 4; Packard Corporation, Downers Grove, IL).

When cocktails of MoAbs were used they were prepared by adding equal quantities (as determined by protein) of each MoAb and then conjugated with radiiodine. The only exception is shown in Fig. 3 where a different approach was used whereby each MoAb comprising the cocktail was radioiodinated separately and then equal μCi amounts of each were added, in order to compare effectiveness of treatment by either method of cocktail preparation. No apparent difference was found with the use of either of these two approaches in therapeutic results (Figs. 3 and 4).

Histopathological studies were performed on paraffin embedded tissue sections stained by immunoperoxidase techniques with MoAbs (5 μg/ml) as already described (7). Standard error of mean and statistical significance were calculated as reported previously using the Student t test (30).

RESULTS

In efforts to circumvent the heterogeneity of antigenic expression of the target cells that interferes with tumor destruction, when using unconjugated MoAbs in experimental therapy of breast cancer (25, 26), MoAbs were tagged with a radioisotope and thus had the opportunity after binding to the cancerous cells to attack the whole tumor mass directly in spite of not being able to bind to every cell. For this purpose, IgGs were purified by high performance liquid chromatography and then tested for sterility and radioiodination with 131I at not more than a 1:1 ratio of 131I to IgG molecules to avoid denaturation. After conjugation 131I-labeled MoAbs were tested for retention of immunological reactivity using delipidated HMFG bound to a solid phase (31) as target. For this purpose, the binding of 131I-conjugated MoAbs to the solid phase was determined, in a double labeling experiment, by its subsequent detection with 125I-labeled goat anti-mouse immunoglobulin. Simultaneously, an identical amount of the same MoAb cocktail, this time unconjugated, was incubated on the solid-phase bound HMFG and again the amount bound was detected by the same 125I-goat anti-mouse immunoglobulin. Comparisons of the binding activity of the 131I-MoAb over the binding activity of the same unconjugated MoAb gave the proportion of immunoreactive conjugated MoAb binding. Binding of the 131I-MoAbs used was in every case above 80% of that of the native unconjugated MoAb.

To test biodistribution of 131I-labeled MoAbs, injections of 4.93 μCi of 131I-Mc5 (specific activity, 2.96 mCi/mg) were given to nude mice grafted with human breast tumors and compared to the injection of 3.73 131I-labeled MoAb mixture (cocktail) comprising MoAbs Mc1, Mc3, Mc5, and Mc8 (specific activity, 3.97 mCi/mg), 3.1 μCi of 131I-Mc3 (specific activity, 6.37 mCi/mg), and 2.83 μCi 131I-labeled Nlg (specific activity, 3.62 mCi/mg). For this purpose, BALB/c nude mice carrying MX-1 tumors averaging 95 mm³ in volume were given i.p. injections and five mice in each injected group were sacrificed at days 1, 4, 6, and 8. In Fig. 1, results of this experiment can be seen where the percentage total dose/g of tissue declined in blood throughout the period as well as in every organ studied, with the exception of the transplanted human breast tumor MX-1. Only in the case of Nlg did the percentage total dose/g tissue decline steadily throughout the period for MX-1 tumor. Statistically significant higher values for incorporation of 131I-conjugates of Mc3, Mc5, and cocktail into MX-1 tumor versus the incorporation of 131I-Nlg were found at day 1. In addition, at day 1 other tissues of the hosts had incorporation of the three types of labeled conjugates which were statistically significantly higher from 131I-Mc5 with the exception of stomach, brain, and muscle for Mc3 and muscle for Mc5. By day 4 the highest incorporation of Mc3 and cocktail (8.5 and 4.5%/g of dose, respectively) of MX-1 tumor was observed and for Mc5 only a small decline from day 1 (3.8 versus 3.2%/g of dose, not statistically significant); in contrast, incorporation into other tissues of the hosts declined in a parallel fashion with blood. The doses of Mc3 and the cocktail incorporated by tumor MX-1 were higher than that of MoAb Mc5 and remained statistically...
To study whether free $^{131}I$ would contribute substantially to the values of tissues of uptake found in Fig. 1, a biodistribution of free $^{131}I$ was performed in BALB/c nude mice either blocked by prior injection of 2 mg potassium iodide or not blocked (Fig. 2). During the time of the biodistribution (1–8 days) blocking for uptake of free $^{131}I$ was most effective in skin and thyroid when compared to nude mice unblocked. MX-1, liver, kidney, spleen, blood, stomach, intestine, bone, brain, lung, and muscle were also found reduced. Unblocked values of tissue uptake were somewhat higher than those of blocked values for free $^{131}I$; however, both of them were substantially lower than those levels of incorporation shown for Mc3, Mc5, cocktail, and Nlg in Fig. 1. At day 1 all levels of tissue uptake in Fig. 2 were below 0.1% total dose with the exception of stomach and thyroid. The unblocked thyroid accumulated 8.8% of the total injected dose whereas the blocked thyroid had 0.0029% of the injected dose. Therefore, even if there is free iodide due to dehalogenation it would be readily excreted and not contribute substantially to...
the whole animal dose or therapeutic dose to the tumor (see later).

The tumoricidal activity of Mc1, Mc3, Mc5, and Mc8 in unconjugated form has been shown for breast tumor (24). To determine the effectiveness of each of these MoAbs radiolabeled, 131I-conjugates of Mc1 which was labeled at a specific activity of 16.27 mCi/mg, Mc3 at 20.10 mCi/mg, Mc5 at 18.72 mCi/mg, Mc8 at 23.42 mCi/mg, and Nig at 12.82 mCi/mg were prepared separately, and the cocktail mixture was prepared with equal amounts of radioactivity of each labeled MoAb, averaging 19.63 mCi/mg. The single 131I-conjugated MoAbs and their cocktail were injected at day 0 in doses of 500 μCi (Fig. 3). All singly injected 131I-MoAbs affected tumor growth with Mc3 being the most effective. Tumor volumes in mice given injections of the different 131I-MoAbs and their cocktail were smaller compared to the uninjected control and were at a statistically significant level (P < 0.001) except for Mc8 (P < 0.005) at day 11. By day 18 Mc1, Mc5, and Mc8 injected MX-1 bearing nude mice had resumed their growth whereas Mc3 and cocktail had not. In fact, maximal reduction of tumor volume for Mc3 injected nude mice was recorded on day 18 with reduction to 52.4% of day 0 volume (P < 0.005, Mc3 versus control) (Fig. 3). In this experiment a 131I-labeled cocktail of the four MoAbs, composed of equal radioactivity amounts of each MoAb to add up to a total equal 500 μCi, with each MoAb contributing approximately 125 μCi, was injected. This was done since in previous work this same cocktail in unconjugated form was most effective in treating breast tumors. The cocktail treated group had a %IG of 75.6% of day 0 volume by day 6 (P < 0.001, cocktail versus control) and 87.2% at day 18 (P < 0.005, cocktail versus control), but the 131I-Mc3 injected had 48.6 and 90.6%, respectively for days 6 and 18 (Fig. 3). By the termination of the experiment at 34 days all injected groups remained statistically significant below the control, Mc3 (P < 0.025), Mc8 (P < 0.05), and cocktail (P < 0.05) except for Mc1 and Nig.

Day 27 after the injection of the radioconjugate was chosen to calculate the %IG to permit interexperimental comparisons since it corresponded to the latest date when inhibition of growth was in full effect for the most potent 131I-MoAbs and the 131I-cocktail (Table 1).

In view of the large efficiency of inhibition of tumor growth demonstrated by a single injection of 500 μCi of Mc3 (efficiency that was comparable to that of the cocktail), another experiment was performed to test the %IG created by a dose of 131I-Mc3 comparable to the amount which this MoAb contributed to the cocktail. For this purpose, 125 μCi of 131I-Mc3 were injected at day 0 and its inhibition of tumor growth compared to that of 500 μCi of the complete cocktail, which contained the other 3 MoAbs in equal amounts plus MoAb Mc3 (Table 1). As shown in Fig. 4 the 125-μCi dose of Mc3 had some growth inhibition potential when compared to either uninjected (P < 0.025) or 500 μCi of Nig (P < 0.01). However, when compared to cocktail, 125 μCi of 131I-Mc3 had a smaller tumor growth inhibition at a statistically significant level (P < 0.05) at day 34.

To test for effectiveness of therapy on a non-breast carcinoma, nude mice carrying a transplantable colon carcinoma CX-1 were given injections of 500 μCi of an 131I-radioconjugate of MoAb Mc5 or cocktail (Fig. 5). Histological sections had previously shown that Mc5 cross-reacted with CX-1 as shown by immunoperoxidase staining (data not shown). The 131I conjugates of Mc5 and cocktail were labeled at specific activities of 10.75 and 9.95 mCi/mg, respectively. The 131I-Mc5 marginally arrested, but not at a statistically significant level, the growth of the tumor when compared with the untreated control. Cocktail became statistically significant (P < 0.05) at only days 39 and 45 when %IG was 57.6 and 44.7, respectively, when compared to untreated control.

Table 1 Calculation of %IG after the injection of 131I-conjugated MoAbs

<table>
<thead>
<tr>
<th>MoAb or Nig</th>
<th>Injection dose</th>
<th>Days after initiation of treatment</th>
<th>%IG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nig</td>
<td>1st 500 μCi</td>
<td>27</td>
<td>4.95</td>
</tr>
<tr>
<td>Nig</td>
<td>2nd 500 μCi</td>
<td>55</td>
<td>4.30</td>
</tr>
<tr>
<td>Mc1</td>
<td>500 μCi</td>
<td>27</td>
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<tr>
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<td>500 μCi</td>
<td>27</td>
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</tr>
<tr>
<td>Mc8</td>
<td>500 μCi</td>
<td>27</td>
<td>61.3</td>
</tr>
<tr>
<td>Mc3</td>
<td>125 μCi</td>
<td>27</td>
<td>63.6</td>
</tr>
<tr>
<td>Mc3</td>
<td>1st 500 μCi</td>
<td>27</td>
<td>87.1</td>
</tr>
<tr>
<td>Mc3</td>
<td>2nd 500 μCi</td>
<td>55</td>
<td>85.5</td>
</tr>
<tr>
<td>Cocktail</td>
<td>1st 500 μCi</td>
<td>27</td>
<td>89.6</td>
</tr>
<tr>
<td>Cocktail</td>
<td>2nd 500 μCi</td>
<td>55</td>
<td>90.8</td>
</tr>
</tbody>
</table>

* Calculated from Fig. 10A.
" Calculated from Fig. 10B.

To further explore the implications of the biodistribution studies shown in Fig. 2, BALB/c nude mice carrying MX-1 were given injections of 500 μCi of free 131I (Fig. 6), and its therapeutic ability was determined. Although the mean tumor volume of MX-1 tumor was smaller at all time points in mice given injections of 131I when compared to the control, the results...
RADIOIMMUNOTHERAPY OF HUMAN BREAST CARCINOMAS

Fig. 4. Experimental radioimmunotherapy of established and growing transplantaible human breast tumor MX-1 grafted in nude mice, with MoAb Mc3, Nig, and cocktail of MoAbs composed Mc1, Mc3, Mc5, and Mc8. One hundred twenty five μCi of 131I-labeled MoAb Mc3 and 500 μCi of both 131I-labeled Nig and cocktail were injected i.p. at day 0 of the experiment which was the 17th day after grafting. Controls received no treatment. Each point represents 4 animals; bars, SE.

Fig. 5. Experimental radioimmunotherapy of established and growing transplantaible human color tumor CX-1 grafted in nude mice with 131I-labeled MoAb Mc5 and a cocktail of MoAbs composed of MoAbs Mc1, Mc3, Mc5, and Mc8. Five hundred μCi of MoAb Mc5 and cocktail were injected i.p. at day 0 of the experiment which was the 16th day after grafting. Controls received no treatment. Each point represents 4 animals; bars, SE.

Fig. 6. Experimental radioimmunotherapy of established transplantaible human breast tumor MX-1 with free 131I. Five hundred μCi were injected i.p. at day 0 of the experiment which was the 29th day after grafting. Controls received no treatment. Each point represents 4 animals; bars, SE.

were not statistically significant at P < 0.05 for any of these time points.

In order to test the effectiveness of repeated doses of 131I-conjugated MoAbs on the inhibition of tumor growth, a new experiment was planned where at days 0, 4, and 9 131I-Mc5 was injected into nude mice implanted with transplantaible human breast carcinoma MX-1. As controls, a comparable group of animals received 131I-labeled high performance liquid chromatography-purified IgG obtained from ascites prepared by injecting X63B cells, the parent myeloma cell line of MoAb Mc5. Three 500-μCi doses of each conjugate with specific activities of 4.96, 5.98, and 4.32 mCi/mg for 131I-Mc5 or 3.92, 4.79, and 5.79 mCi/mg for 131I-control IgG, were injected i.p. in each experimental group consisting of three animals. The mean initial tumor size was approximately 100 mm³ in both groups. After 11 days, the X63B control group had an average tumor size of 344.0 mm³, while the group given a 131I-Mc5 injection had an average tumor volume of 5.6 mm³ (P < 0.001, Mc5 versus control) (Fig. 7). A total dose of 1500 μCi of 131I-Mc5 had a profound tumoricidal effect as judged by the posttreatment size (%IG = 98.4) and histology (Fig. 8). All mice in both groups given injections of either 1500 μCi of 131I-Mc5 or 131I-X63B died. However, monoclonal antibody specificity is shown by the fact that the tumors of the 131I-Mc5 injected mice continued to grow (344% of original tumor volume at day 11 after initial therapy) whereas the tumors of the 131I-Mc5 injected mice were reduced to 5.3% of the original tumor volume at day 11 after initial therapy (Fig. 7). These latter tumors contained just a few epithelial cells which still bound Mc5 as shown by deep staining with immunoperoxidase, as strong as that of cells in the control tumor and surrounded by collagenous stroma or scar tissue (Fig. 8); in contrast, the tumors in the control group were spared and showed histologically normal cellularity with high levels of expression of the Mc5 antigen (Fig. 8). This demonstrates the ability of 131I-MoAbs to destroy almost completely the neoplastic epithelial cell population of breast tumors once a high enough dose is achieved. The present results could not be attributed to the toxicity and local effects of total body irradiation since these tumors in the mice given injections of
same amounts of $^{131}$I-X63B kept growing and seemed intact histologically.

Next, two alternative protocols were tested in experimental immunotherapy of MX-1 tumors: (a) single versus multiple doses; and (b) different dose levels. To test the latter, four groups of five nude mice, each carrying transplantable human breast tumor, MX-1, were given injections at day 0, respectively, of 125, 250, and 500 $\mu$Ci of radiiodinated MoAb cocktail (specific activity, 8.81 mCi/mg) and no treatment (Fig. 9). Injections of similar doses were repeated for levels of 125 and 250 $\mu$Ci on day 5 (specific activity, 7.13 mCi/mg) and day 12 (specific activity, 9.32 mCi/mg), respectively, while the group receiving 500 $\mu$Ci received only another equal dose on day 12, and the control received no injection. As is shown in Fig. 9, all doses seemed effective in arresting growth of the tumor. Doses of 125 $\mu$Ci at day 0 arrested growth of MX-1 as compared to the control; however, the tumor resumed growth at day 5, and after the second dose of 125 $\mu$Ci administered at day 5 seemed to have a similar effect, and tumor growth continued until day 12 when a third dose of 125 $\mu$Ci was injected and diminished tumor size showing a nadir at day 19. Tumor volume recorded for day 12 was not regained until day 28. From here on, the tumors continued to increase in volume albeit at a much slower rate than the control (Fig. 9). Tumors treated with 250 $\mu$Ci at three consecutive times (day 0, 5, and 12) had their growth inhibited, without resumption of growth (in contrast to that recorded for 125 $\mu$Ci doses), until at least day 28 (Fig. 9). Again in the group injected with 250 $\mu$Ci tumor growth resumed at day 28 but also at a much slower rate than in control mice. Possibly as a result of the accumulated dose received, one animal died in each group receiving three injections of 125 and 250 $\mu$Ci and three animals died in the group receiving two injections of 500 $\mu$Ci; however, no animals perished in the control group. Two consecutive injections of 500 $\mu$Ci, at days 0 and 12, destroyed most of the tumor mass and showed 97.0% at 65 days post-first injection (Fig. 9). No reduction in weight was noticed in any group, with the exception of the group receiving two 500-$\mu$Ci injections, in which case animals started to lose weight 3 days after the second injection. Histopathological review of the tumors treated with 500 and 250 $\mu$Ci showed multiple and extensive foci of necrosis throughout the tumor while tumors in control animals had no such lesions (not shown).

These results (Fig. 9) showed effective tumor destruction but high toxicity with closely spaced doses (12 days). Further, the injection of individual MoAbs, as well as their mixture (Fig. 3), showed that a dose of 500 $\mu$Ci of $^{131}$I-cocktail and a similar dose of $^{131}$I-Mc3 reinitiated growth between 20 and 30 days after their administration. Therefore, injections of repeated similar doses were performed for Mc3 and cocktail, with sufficient time for radiation recovery. The second injections of 500 $\mu$Ci of $^{131}$I-cocktail (Fig. 10A) and of $^{131}$I-Mc3 (Fig. 10B) were given at 35 and 21 days after the first injection, respectively. Nude mice carrying established MX-1 breast tumors 300 mm$^3$ in mean volume were injected on day 0 with either NIg or a cocktail of MoAbs labeled with $^{131}$I at specific activities of 5.25 and 7.47 mCi/mg, respectively (Fig. 10A). A group of untreated tumor bearing mice were used as a control. As shown in Fig. 10A the second injection of 500 $\mu$Ci of $^{131}$I-cocktail, given at 35 days after the initial one, again reduced tumor volume and inhibited growth. Tumor growth was inhibited by this second dose for at least another 20 days (Fig. 10A), after which the tumor growth was reinitiated albeit at a much slower pace than both the group receiving similar doses of $^{131}$I-labeled normal mouse IgG and the control. Note that the tumor growth inhibition obtained after $^{131}$I-cocktail conjugate injection was compared after both doses and also that the cumulative effect of both doses attained a 90.8% IG (Table 1). Similarly doses of 500 $\mu$Ci of $^{131}$I-Mc3 at a specific activity of 11.62 mCi/mg were injected into a group of mice grafted with MX-1 tumor, 220 mm$^3$, at day 0. Uninjected mice carrying MX-1 served as controls (Fig. 10B). This first injection was followed 21 days later by a similar one (specific activity, 3.83 mCi/mg). Here again, a prolonged inhibition of tumor growth was obtained (up to 85.5%) which lasted at least another 20 days after the second dose (Fig. 10B; Table 1). Note that the tumors in the groups receiving $^{131}$I-NIg and the untreated mice (control) grew at similar rates (Fig. 10B), demonstrating the specificity of the MoAb cocktail and also that the dose of radioactivity used had no effect on the breast tumor by whole body irradiation. No toxicity or mortality was recorded among the animals in either group thus demonstrating the importance of allowing enough recovery time between multiple doses.

**DISCUSSION**

In previous experiments (24) the same MoAbs used in this study were administered unconjugated to successfully treat breast tumors in nude mice. Total dose of unconjugated MoAbs administered amounted to large quantities by the end of treatment (24), and best results were obtained with a mixture of four unconjugated MoAbs in equal parts than with the use of each MoAb alone. Here these same MoAbs were used radioiodoconjugated and proved to be again effective in breast tumor size reduction and in sustained inhibition of growth.

In the present study these same MoAbs as a cocktail, and in either one or two doses of 500 $\mu$Ci were effective in destroying tumor mass and inhibiting growth of established breast tumors in nude mice for up to 55 days. Here, however, one of the MoAbs used (Mc3) at the same dose level as the $^{131}$I-labeled cocktail, was as effective, in contrast to the previous studies using unconjugated MoAbs (24), where Mc3 was not as effective.
as the cocktail. These MoAbs were radioiodinated without loss of binding ability and, as a result of it, therapeutic results on the breast tumors were better than those obtained with the unconjugated MoAbs above using approximately 150 times less MoAbs.

The duration of the inhibition in growth and the diminution of tumor volume obtained with radioiodinated MoAbs are more profound than those obtained with unconjugated MoAbs (24), indicating that possibly a more diverse population of tumor cells is affected, with actual acute and substantial killing of target tumor cells. The latter is shown by large %IG and the almost total elimination of tumor cells in the tumor remnants after MoAb radioiodoconjugate treatment when studied at the histopathological level (Fig. 8). In that same experiment the tumors in the control group receiving $^{131}$I-labeled IgG fraction of X63B ascites at the same dose level continued to grow, and their mass was composed of breast epithelial cells (Fig. 8).

In similar studies, where a small percentage of tumor cells survived radioiodolabeled MoAb treatment, the surviving tumor cells later repopulated slower growing tumors that could be again successfully attacked by $^{131}$I-MoAbs (Fig. 10; Table 1). This second dose of radioiodoconjugate again decreased tumor volume and inhibited growth for a comparable period, implying that possibly a similarly diverse cell population was available to be attacked (Fig. 10), as shown by the binding of Mc5 by breast epithelial cells surviving intense treatment as shown in Fig. 8, C and D. In contrast, in previous experiments administration of the same MoAbs, unconjugated, resulted in the selection of a cell population which was refractory to treatment and had reduced MoAb binding (24).

The percentage of initial cell killing was very high in breast tumors treated with our radioconjugated MoAbs, such that in spite of local edema and infiltration, reductions of tumor volume of up to 50% are seen at 4 to 5 days after injection (Figs. 3, 7, and 10A, and 10B). Most effective, both at short term tumor destruction and at long term tumor growth inhibition, were $^{131}$I-Mc3 and the $^{131}$I-cocktail. The effect obtained with the latter could not be attributed only to the action of $^{131}$I-Mc3, which contributed 25% of the composition of the cocktail (Figs. 3 and 4; Table 1). It must also be noted that the other MoAbs contributing to the cocktail had substantial MX-1 tumor uptake (23) and had demonstrated MX-1 tumor Ig effect when administered $^{131}$I-conjugated (Fig. 3).

The large short term destruction of breast tumor and prolonged inhibition of growth obtained with both cocktail and single $^{131}$I-conjugated MoAbs took place in the presence of high levels of circulating human breast epithelial antigens in the nude mice grafted with human breast tumors (32). In the breast cancer patient circulating breast cancer antigens (9, 33), as well as their immune complexes (34) are found as we have already reported, and thus in the case of using the present approach in the clinical setting the presence of these circulating antigens will have to be taken into account.
Radioimmunotherapy of human breast carcinomas

Fig. 9. Experimental radioimmunotherapy of established transplantable human breast tumor MX-1 grafted in nude mice with 3 i.p. doses (at days 0, 5, and 12 of the experiment, day 0 being the 18th day after grafting) of 125 and 250 µCi and 2 i.p. doses (at days 0 and 12 of the experiment) of 500 µCi of a 131I-labeled cocktail of MoAbs composed of MoAbs Mc1, Mc3, Mc5, and Mc8. The control received no treatment. Numerator of ratio indicates surviving animals at day 75 of the original number in the denominator. Bars, SE.

These MoAbs at the dose chosen for testing efficacy of breast tumor therapy (500 µCi) seemed to be tumoricidal and tumo-rostatic as a result of binding to the tumor as shown by the fact that a similar dose of radioconjugate of normal mouse IgG was not effective in tumor destruction or inhibition of growth (even when compared to the un.injected control) (Figs. 3, 4, and 10). Neither were 500 µCi of free 131I (Fig. 6). Thus, it is established that these 131I-MoAbs destroy breast epithelial cells contained in the tumor without mortality to the nude mouse host.

A favorable feature of 131I-Mc3 and the 131I-cocktail of MoAbs used in these studies is their prolonged tumor residence time. Eight and one-half and 4.5 of % dose/g of tissue respectively, of 131I-Mc3 and 131I-cocktail were present at day 4 on the tumors and 5.6 and 1.7% were still retained at 8 days (Fig. 1). Thus, the choice of a radioisotope with a relatively long physical half-life like 131I seems justified; however, the particle mass and energy [electron decay, energy (mean) = 202 kev; γ-ray, energy = 364 kev] of 131I are still not entirely ideal. Free 131I will be quickly voided from the experimental host (especially when the thyroid is blocked), as shown by our biodistribution studies (Fig. 2), which indicated that dehalogenated 131I does not contribute much to either whole body or tumor irradiation. These added considerations also support further the use of 131I under the present circumstances.

The therapeutic effectiveness of the 131I-labeled MoAbs is dose dependent (Fig. 9), reaching a level, after 3 repeated injections of 500 µCi each, where most if not all live epithelial tumor cellularity is eliminated (Figs. 7 and 8). However, this total dose of 131I-cocktail is highly toxic. Thus, approaches that increase accumulation of 131I-conjugate on the tumor (more efficient MoAbs, better capillary permeability within the tumor, augmentation of cell-per-cell expression of target antigen, increasing number of cells expressing the target antigen, more complete cocktails comprising more MoAbs, etc.) could decrease the total dose required. Of those choices available, induction of target antigen expression and more complete cocktails of MoAbs could be the most attainable ones. Alternatively, radiiodoconjugate doses of MoAbs above maximal tolerated dose and with total tumoricidal effectiveness could be administered if supported by posttherapy bone marrow infusions.

The value of enough time for recovery from high level tumoricidal doses is shown when comparing the toxicity after 2 doses of 500 µCi 131I-MoAbs separated by 12 (Fig. 9) and 35 (Fig. 10) days, respectively, the former very high, the latter negligible, while the effect in terms of tumor destruction was not much different. Having enough time for recuperation from radiation damage after a 131I-MoAb injection, will be more possible in human breast cancer patients, since breast tumors in the patient have a slower growth than the ones used in this work, and repeated MoAb radioconjugate doses could be separated by time periods of month.

Tumor attack by MoAb radioconjugates in immunodeficient hosts (as the present ones) could well be very different from...
that occurring in an immunocompetent host. Although a potentially interfering immune response to the foreign immunoglobulin could arise, cellular reaction to the tumor in the immunocompetent host could simultaneously enhance the radioimmunotherapeutic effects.

The successful experimental treatment of human breast tumors with radioiodoconjugated MoAbs presented here with both effective tumor destruction and sustained inhibition of tumor growth warrants further exploration of the therapy of breast cancer with radioiodoconjugated MoAbs.

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