Experimental Immunotherapy of Human Breast Carcinomas Implanted in Nude Mice with a Mixture of Monoclonal Antibodies against Human Milk Fat Globule Components

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ABSTRACT

Immunological therapy of BALB/c nude mice (nu/nu) implanted with human breast tumors, estrogen receptor negative MX-1 and estrogen receptor-positive MCF-7, was carried out with four monoclonal antibodies (MoAbs) raised against human milk fat globule membrane glycoproteins also present on normal breast epithelial cells. MoAbs injected singly or as a partial mixture arrested growth of the tumors but to a lesser extent than a mixture ("cocktail") of all four MoAbs. Two model systems were developed in order to examine the capabilities of the four MoAbs to arrest human mammary tumor growth. In the first model the ability of these MoAbs to arrest tumor growth during a 6- to 8-week period was tested by injection of the MoAbs immediately before and after implantation (passive immunization) and thereafter every other day. In the second model the effect of these MoAbs on established and growing tumors was tested. Using the cocktail in the passive immunization protocol, human mammary tumor growth in nu/nu mice was arrested either completely or averaging to one-tenth the size of the controls for those mice in which the tumors had taken. Other human carcinomas, colon and lung, under the same protocol, were not affected. Injection of cocktail every 2 days into nu/nu mice with established and growing human breast tumors (both estrogen receptor positive and negative) produced arrests of tumor growth of 44.1, 45.2, 49.8% of their controls after 7 to 8 days of treatment.

Previously, it has been established that human mammary tumors are heterogeneous in expression of the human milk fat globule antigens recognized by our antibodies to the extent that some cells may have large amounts and others no detectable amount of a particular antigen. Those MX-1 tumors treated for a prolonged time with the cocktail of MoAbs that survived and continued to grow could be the result of the preferential multiplication of those cells in the heterogeneous population which had low or no antigen content. The breast tumors that did grow in the nu/nu mice after 8 weeks of injection of the cocktail revealed by immunoperoxidase staining a 90% reduction in the antigen content as recognized by our antibodies to the extent that some cells may have large amounts of a particular antigen. Those MX-1 tumors treated for a prolonged time with the cocktail of MoAbs that survived and continued to grow could be the result of the preferential multiplication of those cells in the heterogeneous population which had low or no antigen content. The breast tumors that did grow in the nu/nu mice after 8 weeks of injection of the cocktail revealed by immunoperoxidase staining a 90% reduction in the antigen content as recognized by these MoAbs when compared with untreated tumors. These results attest to the effectiveness of unconjugated anti-human milk fat globule MoAbs to arrest human breast tumor growth in nu/nu mice, and they also suggest that to best arrest tumor growth the use of a mixture of MoAbs should be considered.

INTRODUCTION

The use of MoAbs3 with tumor specificity for the therapy of breast tumors becomes debatable in view of the few, if any, true high prevalence breast tumor antigens found to date (1). Obviously, the alternative to such a dilemma is the use of MoAbs that recognize normal antigens of the breast. In this case, the differentiated phenotype of a given tumor sustained after transformation becomes the target. However, these anti-HMFG MoAbs do not have a specificity restricted to human breast epithelial cells alone. By immunoperoxidase staining of fixed and frozen sections, Mc1 recognized luminal surfaces of human liver, sebaceous gland, kidney, sweat gland, epididymis, uterus, acinar cells of pancreas, exocrine serous demilunes of the minor salivary gland, and the cell surface and intracellular globules of the sebaceous gland (2); Mc5 recognized human cervix, endometrium, kidney, liver, lung, ovary, pancreas, stomach, and thyroid; Mc3 stained ductal breast carcinomas (13 of 37) but failed to stain lobular (0 of 6), medullary (0 of 1), comedo (0 of 1), mucinous (0 of 1), tubular (0 of 2), adenosquamous (0 of 1), fibroadenoma (0 of 2), and normal breast (0 of 1). In addition, Mc3 failed to recognize the following tissues: cervical, duodenal, endometrial, renal, ovarian, pancreatic, prostatic, thyroïd, and colonic adenocarcinomas, as well as hepatoma, lung carcinoid, and mesothelioma. Mc8 stained ductal breast carcinoma (10 of 37), but failed to recognize lobular (0 of 6), medullary (0 of 1), comedo (2 of 2), mucinous (0 of 1), tubular (0 of 2), normal (0 of 1), and adenosquamous (0 of 1), and fibroadenoma (1 of 2). In addition, Mc8 stained 1 of 8 renal adenocarcinomas but failed to stain cervical, colonic, duodenal, endometrial, lung, ovarian, pancreatic, prostatic, parathyroid, and stomach adenocarcinomas, as well as hepatoma, mesothelioma, and lung carcinoid.

To examine the issue of anti-HMFG MoAbs cross-reactivity with human tissues other than breast, we have proposed the use of nu/nu mice implanted with human non-breast tumors which have retained their differentiated phenotype (3), thus acting as different control human tissues. This model is advocated since cross-reaction of mouse MoAbs with human tissues and not mouse tissues is the point of interest and whereas in vivo human testing would not be convenient.

The use of MoAbs directed to normal differentiation antigens is limited to tumors of certain organs whose original cells are not indispensable for the survival or anatomic integrity of the patient under treatment. In this category, by definition, fall tissues like the prostate and breast. These two glands are the sites of very common carcinomas in males and females. This fact adds even more emphasis to the targeting of antigens with restricted tissue distribution in experimental tumor immunology. This approach was thus taken when we prepared a series of MoAbs binding HMFG antigens but not the membrane antigens of the epithelial cell lines, HT-29 and HeLa, Bristol-8, a lymphoma cell line, or normal human fibroblasts (1, 4, 5). These MoAbs that recognize antigens with high prevalence in human breast tissue (6), both normal and neoplastic, have already been successfully used by us in an experimental immunotherapy model in efforts to arrest breast cancer growth (7).

In preliminary experiments, nu/nu mice implanted with a transplanted human breast carcinoma and simultaneously given injections of one or several of these MoAbs, not only survived much longer than the noninjected ones, but after 8 weeks carried tumors only 10–20% the size of the controls (7). Further non-breast tumors CX-1 (colon) and LX-1 (lung) were not affected. Efforts in this direction by another laboratory later confirmed that with another such MoAb it was also possible to arrest the growth of another transplantable breast tumor in the...
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nu/nu mouse system (8). In both of these attempts at experimental immunotherapy of breast tumors, the MoAbs were injected unconjugated.

It has been previously proposed that MoAbs conjugated with toxins could have enhanced cytotoxic effect (9). Nevertheless, in view of the success obtained with unconjugated MoAbs, both by us and by others, we decided to explore their use systematically in an experimental model system. Thus, in this paper we propose the use of MoAbs against normal high prevalence breast epithelial components in the experimental immunotherapy of breast cancer. Two approaches are presented here, the one whereby the MoAbs are given as passive immunization and the other where MoAbs are given as direct treatment for already established tumors.

It can be seen that these two approaches are valid. In the first one, the therapeutic MoAbs are injected simultaneously with the graft of the breast tumor or previous to this event, thus introducing a passive immunization to the breast tumor implantation. The scenario will be the one in which MoAbs will be used to destroy nonimplanted, possibly circulating, breast tumor cells and/or interfere with their attachment and lodging into vital organs. This case will be the one in which hampering of metastatic dissemination and tumor cell lodgment occur, either produced by spontaneous release of tumor cells or as a result of surgical intervention. In this latter case, passive immunization is proposed as a type of mopping up of the surgical field. Alternatively, and possibly closer to the everyday oncological situation, the second approach consists in the use of MoAbs in their direct attack of the already established experimental breast tumor.

Different parameters that represent biological functions of the tumor must be measured to characterize fully the activity of MoAb treatment. In the case of passive immunization with anti-HMFG MoAbs, not only the date of first appearance of tumor, survival of tumor-bearing mice versus controls, tumor size, and histopathological appearance of tumor, but number of successful grafts in the presence of circulating MoAbs should be taken into account. In the latter case of immunotherapy of established tumors, direct measurement of the tumor mass, survival of the tumor-bearing mice (the MoAb-treated mice versus their controls), and histopathological review of the effect of the MoAbs on the tumor mass are desirable parameters to be measured. In addition, in either approach, quantitative determinations of immunohistochemical staining of the corresponding phenotypic expression of the antigens on the treated and untreated tumor cell population should be performed in an effort to understand the resistance that could develop to this form of therapy and to envision solutions to this problem.

Although in one study (8) only one MoAb was used to arrest human breast tumors in nu/nu mice, the constant trend of all tumors to produce phenotypic variants (10) could possibly interfere with its complete eradication. Human breast tumors have been specially studied for their heterogeneity of antigen expression and the rate at which antigens detected by anti-HMFG MoAbs vary randomly (11, 12). From these studies it was evident that there will always be a cell subpopulation in expression and the rate at which antigens detected by anti-HMFG MoAbs and mixtures thereof. Efforts were also made to evaluate the effect of MoAb treatment at the cellular level and the result of such treatment on the different tumor cell populations.

MATERIALS AND METHODS

Nude mice (nu/nu) with a BALB/c background were purchased from Life Sciences, Inc. (St. Petersburg, FL). Female nu/nu mice were grafted with transplantable human tumors at 7 to 8 weeks of age (body weight, 19 to 24 g). They were placed in sterile cages with sterile bedding, one per cage, provided Purina mouse chow (5015) and acidified (pH 2.5) water. nude. Grafts were steriley implanted through a flank incision midway between front and hind legs. The 1-mm-diameter graft was then gently pushed above the hip with a fine forceps. The wound was closed with metal clips.

The transplantable human tumors, estrogen receptor negative (13) MX-1, human breast carcinoma, CX-1, human colon carcinoma derived from HT-29 colon carcinoma cell line, and LX-1 derived from a metastatic lung carcinoma (human lung carcinoma), were obtained from the EG&G Mason Research Institute and have been stereily transplanted in our laboratory for 4 years. The estrogen receptor positive (14, 15) tumor MCF-7 was derived in our laboratory from injection of approximately 30 x 10^6 cells grown in tissue culture, into estrogen-primed female nu/nu mice. Each mouse was primed with a 17β-estradiol 0.5-mg pellet (Innovative Research of America, Rockville, MD) implanted in the interscapular area 2 days before tumor implantation and at 21-day intervals thereafter. For the experiments where MoAb treatment was administered to already established tumors, the tumors and their initial volumes were, respectively, 20 mm^3 and 80 mm^3 for MX-1, 70 mm^3 for MCF-7, and 75 mm^3 for CX-1 non-breast tumor. Measurements of tumor growth in every case were performed with calipers, measuring externally three dimensions, one was the widest diameter, another one, the narrowest diameter, and the third one, the height of the tumor. To obtain volume the formula for the volume of an ellipsoid was used (volume = \(\frac{4}{3}\pi a^2 b\), where a is the largest radius and b is the average of 2 smaller radii).

The MoAbs used were those already described by us (4). Briefly, they were prepared against HMFG membrane glycoproteins (6) by injection into BALB/c mice and hybridization of their spleen cells with a mouse myeloma cell line (4). The hybridomas obtained were screened for positivity for HMFG and for negativity for cervical and colon carcinomas, normal human fibroblasts, and human lymphoid cells (4). The hybridomas of MoAbs used to produce ascites were injected into 2,6,10,14-tetramethylpentadecane-stimulated BALB/c mice with either hybridomas of MoAbs Mc6, Mc3, Mc5, Mc8, or the X63A myeloma cell line. These MoAbs detected, respectively, HMFG antigens with apparent molecular weights of 400,000, 46,000, 400,000, and 46,000 (4). Once ascites was drawn it was spun for 5 min at 200 x g, the supernatant was then spun at 1 x 10^4 x g in a refrigerated centrifuge for 10 min, and was finally filtered through a 0.2-μm Nalgene filter. Then the MoAbs were titered by end point dilution on 18-ng solid phase bound HMFG as already described (16). As a control the asci tes of X63A, the parent myeloma cell line, was used as well as normal mouse serum, as indicated. Normal mouse serum was obtained from BALB/c mice of either sex. Both control ascites and serum were processed and sterilized as the MoAb ascites.

For the MoAb distribution experiments, MoAbs and control normal mouse immunoglobulins were iodinated and injected into nu/nu mice harboring transplantable human tumors, as already described by us (17). At the times indicated mice were bled and the tumors and different organs were dissected and measured for [125I] activity. In the experiments the mixture of MoAbs, the “cocktail,” and normal mouse immunoglobulins were injected, and at the times indicated mice were bled and serum samples were diluted to 1/1000 and measured for circulating antibody titer in an immunoassay comprising its binding to microtiter plates containing 18 ng of HMFG using a [125I]-labeled secondary goat anti-mouse F(ab')2 to detect this binding, as already described (16).

For the passive immunization experiments the nu/nu mice were given injections i.p. of ascites containing 600 μg of MoAb IgG or X63A IgG, or 600 μg of IgG of normal mouse serum. In the case where mixtures of MoAb were injected, each dose contained 600 μg of MoAb.
and an equal partial quantity of ascites IgG for each MoAb used. Then, 24 h after, the tumors were grafted and injections were continued every other day for up to 8 weeks for a total of 16.8 mg/mouse for the duration of the experiment. Alternatively, the MoAb injections were administered immediately after the tumor grafting and injected every other day as before. Either injection schedule is specified for each experiment. Once a visible tumor was discerned caliper measurements were started, and mice were weighted simultaneously with every MoAb injection. When required for histology tumors were excised and fixed in Bouin’s fixative or 12% formalin in phosphate-buffered saline (18).

To determine action of MoAbs on already established human tumors, once the tumors acquired the required size the mice began to be given injections of 2.4 mg of MoAb cocktail ascites or normal mouse serum IgG, a total of 9.6 mg/mouse in a 7- to 8-day period. The former was composed of equal amounts of each of 4 MoAbs used to make up 2.4 mg. In these experiments 4 times the dose per injection of MoAbs was used as a result of preliminary experiments that showed this dose to be most effective. Injections together with animal body weight determinations were performed every other day for the length of the experiment.

Paraffin-embedded tumors were stained histologically by using an indirect immunoperoxidase technique (19). To stain 50 µl of serum-free media from tissue culture grown hybridomas of either the cocktail of the four MoAbs or X63A, the parent myeloma line containing 5 µg/ml of MoAb were used.

Antigenic content of paraffin-embedded sections after immunoperoxidase staining were performed with a microspectrum analyzer (Farrand Optical Co., Valhalla, NY), as already described (11).

RESULTS

Growth of human breast tumors MCF-7 and MX-1, as well as colon (CX-1) and lung (LX-1) carcinomas implanted s.c. in homozygous nude mice (nu/nu) with a BALB/c background proceeded unchecked after their implantation as a 1-mm-diameter tumor graft. At 8 weeks tumor sizes ranged from average values of 600–4656 mm³, and they impaired survival of the mice.

Human tumors grafted in nu/nu mice grew in a comparable fashion. Lung carcinomas, however, had a 4.31 times faster growth than MX-1 and 3.22 times faster than CX-1 when tumor-grafted mice were given injections of normal serum as a control group (Fig. 1). Usually tumor growth was detected at the end of 2 weeks. Starting at this date it was usually easy to measure the three diameters to be averaged, necessary for our tumor volume determinations. Further, it was only after the breast tumors attained 500 mm³ in volume that its innermost areas began to become necrotic. Injections i.p. of a MoAb cocktail of ascites and control sera did not offer any special risk to the nu/nu mice as long as they were appropriately sterilized by filtration. High levels of activity of circulating MoAbs were measured at 5 h, and the rate of disappearance from circulation was about 2 days (Fig. 2).

In the first approach of passive immunization the number of tumor takes (indicating in the experimental groups the ability to interfere with lodgment of a metastatic embolus), and the rate of tumor growth were measured. In the second approach of immunotherapy of an established tumor, rate of tumor growth was the main parameter measured. In addition, in either of the two approaches above, it was found valuable to follow the experimental immunotherapy with immunohistochemical and histopathological study of the tumor itself. From these histopathological studies it was possible to ascertain the amount of tissue destruction and the antigenic profile of the cells surviving the immunotherapy procedure instituted.

In view of the breast tumor cell heterogeneity of antigen expression (11, 12), the alternative presented was whether to inject only one MoAb or two or more of them. To study this question, groups of nu/nu mice were given injections 24 h after...
grafting and every other day thereafter until the end of the experiments of 600 μg of MoAb, obtained from ascites fluid: Mc1, Mc3, Mc5, Mc8 (each separate), or a mixture or cocktail of the preceding 4 MoAbs (consisting of equal amounts of each MoAb), or normal mouse serum or were given injections of nothing (Fig. 3). The amounts of MoAbs injected were always 600 μg, and in the case where mixtures were injected the 600 μg was made up of equal amounts of each ascites. When a single MoAb was injected the total volume of 600 μg was made up of this MoAb only. Tumors became evident at the end of 2 weeks in the normal serum group and noninjected control group, and at 4 to 6 weeks, if at all, in cocktail ascites-treated group. Tumor take was 100% in all groups with the exception of the cocktail group, where take was only 62.5% (Table 1) when these results plus those of 4 other experiments are combined. The two control groups, normal serum and the noninjected control, grew as predicted with their approximate doubling time for volume of approximately 1 week.

In contrast, the rate of growth of MX-1 tumor was altered by the injection of MoAbs (Fig. 3). The most effective MoAb injected alone interfering with tumor growth was Mc8 and the least effective was Mc3. MoAbs Mc1 and Mc5 seemed of similar potency. When the MoAbs were injected as a mixture or cocktail the effect was far superior, achieving a tumor growth inhibition such that at 6 weeks the breast tumors were only 10% the size of those of the controls. This reduced tumor size was a result of a much slower increase in breast tumor growth in the cocktail-treated versus the controls, which was, respectively, 3.88 and 40.47 mm³/day (Table 2). Once the injection of cocktail was stopped, the tumors resumed growth, albeit at a slower rate than the controls. These results showed the ability of the cocktail of MoAbs to inhibit the growth of breast tumors.

In a comparable experiment in an effort to rule out the possibility that the effect of the cocktail on the MX-1 breast tumor could be due to just the action of its most active MoAbs as shown in (Fig. 3). MoAbs Mc5 and Mc8 were used together and compared both to the cocktail and to normal serum injection (Fig. 4). Mc5 and Mc8 did not have the full effect of the cocktail, which in this experiment again inhibited above 90% growth of the MX-1 breast tumors, Mc8 together with Mc5 only inhibited tumor growth by 50%.

The specificity of the MoAbs used in this study was described elsewhere (4), using a solid phase methodology. This procedure, although quantitative and precise, relies on a solid phase con-

Table 1 Percentages of takes of transplantable human tumor implants in BALB/c nu/nu mice given injections of 600 μg of IgG of cocktail consisting of either equal amounts of ascites of MoAbs: Mc1, Mc3, Mc5, and Mc8, 600 μg of normal serum IgG, or no treatment

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Treatment</th>
<th>% Tumor takes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX-1</td>
<td>Cocktail</td>
<td>62.5</td>
</tr>
<tr>
<td>MX-1</td>
<td>Normal serum</td>
<td>100.0</td>
</tr>
<tr>
<td>MX-1</td>
<td>None</td>
<td>100.0</td>
</tr>
<tr>
<td>CX-1</td>
<td>Cocktail</td>
<td>100.0</td>
</tr>
<tr>
<td>CX-1</td>
<td>Normal serum</td>
<td>100.0</td>
</tr>
<tr>
<td>CX-1</td>
<td>None</td>
<td>100.0</td>
</tr>
<tr>
<td>LX-1</td>
<td>Cocktail</td>
<td>100.0</td>
</tr>
<tr>
<td>LX-1</td>
<td>Normal serum</td>
<td>100.0</td>
</tr>
<tr>
<td>LX-1</td>
<td>None</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Number of tumor takes over total experimental nude mice.

Table 2 Average increase in tumor volume of MX-1 growing in untreated and BALB/c nu/nu mice given injections of ascites immediately after grafting and every other day thereafter

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean increase (mm³/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocktail</td>
<td>3.88</td>
</tr>
<tr>
<td>Mc8</td>
<td>6.30</td>
</tr>
<tr>
<td>Mc5</td>
<td>7.57</td>
</tr>
<tr>
<td>Mc1</td>
<td>12.61</td>
</tr>
<tr>
<td>Mc3</td>
<td>27.25</td>
</tr>
<tr>
<td>Control</td>
<td>34.80</td>
</tr>
<tr>
<td>X63A</td>
<td>40.47</td>
</tr>
</tbody>
</table>

Fig. 3. Tumor volume increases after implantation and follow-up to 6 weeks, of MX-1, a human breast carcinoma, grown in BALB/c-nu/nu mice, which were given injections immediately after grafting and every other day of either nothing (control), or 600 μg of ascites of either MoAbs: Mc1, Mc3, Mc5, Mc8, or cocktail consisting of equal amounts of all four MoAbs or the ascites of X63A, the parent myeloma line. Points, means of three animals; bars, SE.

Fig. 4. Tumor volume increases after implantation and follow-up through 8 weeks of MX-1, a human breast carcinoma, grown in BALB/c-nu/nu mice, which were given injections every other day of 600 μg of either an ascites consisting of equal 600-μg amounts of two MoAbs: Mc5 and Mc8, a cocktail consisting of equal amounts of four MoAbs: Mc1, Mc3, Mc5, and Mc8, or the ascites of X63A, the parent line myeloma. Points, means of four tumors; bars, SE.
jugated antigen with its inherent detrimental effect on antigen denaturation and interference with antibody binding. To test experimentally this specificity, nu/nu mice were implanted with human breast carcinoma, MX-1, and a non-breast human carcinoma, CX-1, a colon carcinoma, and distribution studies were performed. For this purpose a G-200 purified IgG fraction of the MoAb cocktail was radioiodinated and injected into the tumor-bearing mice for distribution studies. After subtracting for percentage of dose of normal mouse IgG, it was found that the human breast carcinomas concentrated 4.9% of the dose administered of the labeled cocktail, while the colon carcinoma only retained 0.04% (Table 3).

The specificity of tumor cell killing was then demonstrated in an experiment where nu/nu mice were implanted with human breast carcinoma MX-1 and two non-breast human carcinomas: LX-1, lung carcinoma, and CX-1, a colon carcinoma, and the same protocol as above (passive immunization) was applied. All types of tumor-implanted mice were given injections of either MoAb cocktail or a corresponding amount in protein of normal mouse serum. For mice given injections of cocktail human breast tumor, take rates were 75%; however, they were 100% for both human lung and colon carcinomas. All control groups had 100% take. The rates of tumor growth of cocktail injected versus control were practically identical in both the human lung and colon carcinomas, while the cocktail seriously impaired the growth of the human breast carcinoma when compared to its control (Fig. 1). Uninjected control human breast tumors (MX-1) acquired sizes averaging 14.7 x 12.7 x 9.7 mm and normal serum injected 11.5 x 10.2 x 8.2 mm, whereas cocktail-injected tumors only reached 4.2 x 4 x 3 mm at the end of 8 weeks; typical mice are shown in Fig. 5A. Comparably, cocktail-treated CX-1, human colon carcinoma, and LX-1, human lung carcinoma, grew unimpeded and reached average dimensions of 12.8 x 11.2 x 8.5 mm and 27.2 x 20.6 x 15.5 mm, respectively; typical mice are shown in Fig. 5B at 8 weeks. This study shows the specificity of the cocktail for breast tissue when compared to CX-1 (colon) and LX-1 (lung) and confirms its tumorstatic ability on the breast.

In spite of the prolonged schedule of injections (8 weeks), and the substantial amount of ascites injected (600 µg/mouse/day injection, 16.8 mg/mouse total) no apparent toxic effect was noticed in the mice. The mature nu/nu mice used in these experiments maintained, on an average, their weight through the 8 weeks. Further, at the end of the experiment, no appreciable gross anatomical alterations could be detected in the autopsy of the nu/nu mice subjected to 8 weeks of ascites injection.

Once proven that the cocktail of MoAbs inhibits the growth of breast carcinoma grafts in the passive immunization experiments, a more stringent presentation and one closer to the usual clinical presentation of breast cancer was also tested in an effort to show clearly an inhibition of tumor growth. This latter effect can already be noticed in Figs. 1, 3, and 4, and Table 2, where the rate of the growth for those breast tumors implanted in nude mice given injections of the MoAbs was lower than for those tumors carried by control mice. However, the possibility existed that the smaller sizes of the treated tumors at the end of the passive immunization experiments could only be due to a smaller starting volume and not to a slowing of the rate of growth. Smaller starting sizes could be the result of initial cell killing or inhibition of implantation due to the passive immunization provided by the injection of MoAbs before grafting of always identical tumor pieces. Thus, studies were made where the MoAb cocktail was injected into nu/nu mice harboring already established breast epithelial tumors. For these experiments 600 µg of each of the four MoAbs were combined to make 2.4 mg of cocktail of ascites and injected into nude mice carrying estrogen receptor positive and negative transplantable human breast tumors (9.6 mg/mouse, total dose for 7–8 days). Injection of the cocktail was initiated when the mean volume in 4 mice of human breast carcinoma MX-1 reached volumes of 80 mm³ and 20 mm³ in two different experiments. These volumes were attained, respectively, at 24 and 15 days. Injections continued every other day until termination of the experiment. In the experiment where the mean volume of the breast tumors was 80 mm³ at its initiation (Fig. 6A), an increase of

![Figure 5](https://example.com/figure5.png)

**Table 3** Percentage of distribution of total injected dose of 131I-cocktail IgG and 125I-normal serum IgG per g of tissue in BALB/c nu/nu mice grafted with MX-1 and CX-1 transplantable human tumors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>131I-Cocktail</th>
<th>125I-Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX-1 (human breast tumor)</td>
<td>5.43 ± 0.17</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td>CX-1 (human colon tumor)</td>
<td>0.11 ± 0.11</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>Liver (mouse host)</td>
<td>0.12 ± 0.17</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>Kidney (mouse host)</td>
<td>0.45 ± 0.45</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Muscle (mouse host)</td>
<td>0.34 ± 0.10</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Spleen (mouse host)</td>
<td>0.21 ± 0.18</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Brain (mouse host)</td>
<td>0.19 ± 0.14</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

*Mean ± SE.

* P < 0.001, 131I-cocktail versus 125I-normal serum.

**Fig. 5.** A, BALB/c-nu/nu mice 8 weeks after transplantation with 1-mm pieces of human breast tumor (MX-1). On the left is monoclonal antibody cocktail treated, center, normal serum (NS) treated, right, untreated. B, BALB/c-nu/nu mice 8 weeks after transplantation with 1-mm pieces of lung (LX-1) tumor, colon (CX-1) tumor, and 1-mm pieces of human breast (MX-1) tumor and treated with MoAb cocktail.
Fig. 6. Percentage increase or decrease of MX-1, an estrogen receptor-negative human mammary carcinoma, A and B, and MCF-7, an estrogen receptor-positive human mammary tumor, C, and CX-1, a human colon carcinoma, D, established and growing in BALB/c-nu/nu mice which were given injections of 2.4 mg of either a cocktail consisting of equal amounts of ascites of MoAbs: Mc1, Mc3, Mc5, and Mc8, or normal serum (NS) starting at day 0 and then every other day until the termination of the experiment. Points, means of four tumors; bars, SE.

97% in mean tumor volume was obtained at 8 days for the group of breast tumor-bearing mice given injections of normal serum, and of 6% decrease in mean tumor below the starting volume at 8 days for those given injections of the cocktail (Fig. 6A). Thus, the cocktail-treated group had a mean volume at the end of the experiment which was 44.1% of that of the normal serum control. Respectively, in the experiment where starting mean volume of the MX-1 breast tumors was 20 mm³ (Fig. 6B), the increase of the controls in mean tumor volume at 7 days was 177% and in the cocktail-treated group there was 39% increase in mean tumor volume at 7 days (Fig. 6B), which represented an arrest of tumor growth as the experimental group versus the control of 49.8%. These experiments show clearly the ability of the MoAb cocktail to inhibit the growth of the established and growing breast carcinoma by itself. With the intention of testing the ability of our experimental immunotherapy protocol in other breast tumors, estrogen receptor-positive (14, 15) MCF-7 human breast tumors were tested (Fig. 6C). These tumors had a mean tumor volume at the start of the experiment of 70 mm³ and the host mice were given injections in a protocol similar to that of the mice bearing the MX-1 tumor. After 7 days, tumors averaging a 113% increase over the starting volume, while the MoAb cocktail-treated mice bore tumors diminished in volume by 3% below the starting volume (Fig. 6C). The mean volume of the experimental group was of 45.2% of the control group.

As a control experiment, CX-1 (Fig. 6D), the colon tumor whose mean initial volume was 75 mm³, was treated by injection of 2.4 mg of either the cocktail or normal serum IgG (Fig. 6D). This experiment showed that in the case of CX-1 no benefit was derived from injection of cocktail to the mice whose tumors increased by 152% over the starting volume, while those given injections of normal serum increased 158% (Fig. 6D).

Immunohistochemical studies of the tumors that survived cocktail treatment in the passive immunization experiments for 8 weeks when compared to the control tumors, showed different
antigenic expression for those antigens detected by the cocktail of MoAbs. In immunoperoxidase-stained sections, the cocktail-treated MX-1 tumor (Fig. 7A) stained much less than the untreated MX-1 tumor (Fig. 7B). Both controls of cocktail-treated (Fig. 7C) and untreated (Fig. 7D) MX-1 were not stained with the serum-free supernatants of X63-A, the parent myeloma line. When quantitative measurements of antigenic content were obtained with a microspectrum analyzer, the tumors surviving cocktail treatment expressed a very reduced amount (9.7%) of expression of the antigens when compared to tumors borne by the mice given injections of normal mouse serum in this same protocol (Table 4).

DISCUSSION

The action of unconjugated anti-HMFG MoAbs on human breast tumor growth in nu/nu mice is clearly demonstrated. Their action was shown in two different protocols. In the first one of passive immunization, the grafting of the tumors was shown to be interfered with by the MoAbs, and also their rate of growth was significantly reduced. This effect on the grafting could represent the power of this cocktail to interfere with lodgment of distant or local metastases. In the case of local metastases this could indicate for them a role in “mopping up” after a surgical procedure on the tumor area. This quality becomes more relevant in the more conservative contemporary surgical approaches. Also, the action of passive immunization in diminishing the rate of growth of the tumor when compared to its control is clearly shown, as demonstrated by the diminishment of the rate of growth of those tumors treated with the MoAb cocktail when compared to the controls. Two other pieces of evidence support the action of these MoAbs at the cellular level. First, different MoAbs have varying ability in slowing down the rate of tumor growth, that was maximal with the cocktail. Second, after the cocktail injections were stopped the breast tumors resumed growth at a much faster pace, although not as fast as the controls (results not shown). Protocols such as this one become a useful tool in dissecting the
MoAb action on breast tumors, especially when coupled to studies where the antibodies are also tested on established tumors.

The tumor growth arrest action of anti-HMFG antibodies is clearly proven for each isolated MoAb and for the cocktail. A relevant fact is that the latter has far more effect than the single MoAbs. These results agree with the concept already described in detail by us (11, 12) of the heterogeneity of breast cell surface antigen expression where no tumor studied contained a population of 100% antigen positive cells. This finding was proven not to be the result of tissue fixation since cloned colonies of these tumors had similar heterogeneity of expression (11, 12).

Thus, by using a cocktail of several MoAbs, the chances of increasing the number of cells destroyed by any of the MoAbs is increased. In fact, most cells of the untreated MX-1 and MCF-7 human breast tumors used in our studies bound the cocktail in immunoperoxidase staining. In spite of the cocktail treatment, however, breast tumors continued alive, although growing more slowly. The question of why they continue to grow, at least in part, can be answered by the remarkable difference in specific antigenic content in those cells in the tumor populations that survived treatment (Fig. 7; Table 4) when compared to the original or the normal serum-treated tumor controls.

This event speaks of either reduction of the antigenic production in these cells as a result of the antibody treatment and/ or most likely by the selection of an antigen-poor treatment-resistant cell population. The immunochemistry studies although not choosing for either, or any other alternative, clearly point out the problems still to be resolved in this type of experimental immunotherapy with regard to tumor heterogeneity or the production of treatment-refractory tumor cell populations as a result of therapy. However, due to the fact that the nu/nu mouse immunodeficient host offers such a neutral test system, the hope can be entertained that in the breast cancer patient, with a competent immune system, that MoAb action on the tumor mass can possibly be aided by the patient’s own immune system.

The use of established tumors in these approaches to experimental immunotherapy certainly prove the ability of MoAbs to act in a setting as near as possible to a clinical one. Two experimental groups consisting of four nude mice grafted with estrogen receptor-negative transplantable human breast tumor MX-1, with mean tumor volumes of 80 and 20 mm³ were given injections of the cocktail of MoAbs and resulted in arrests of breast tumor growth where the final volume was, respectively, 44.10 and 49.8% of their controls at 7 and 8 days, respectively. In a similar fashion, four nude mice were grafted with estrogen receptor-positive transplantable human breast tumor MCF-7 (mean tumor volume, 70 mm³) and given injections for 7 days of the same cocktail of MoAbs, resulting in an arrest of tumor growth of 45.2% of the control group. It must be remarked that in two of three of these experiments, the original tumor size was decreased by the injection of MoAbs. Further, the starting tumor sizes assure us of their complete vascularization and lack of necrotic core. Obviously, the presence of a centrally caseated area in tumors restricts to a percentage the total tumor diameter reduction due to treatment and, therefore, understates the therapeutic effect of the MoAbs.

In this study anti-normal differentiation antigens antibodies were used, as represented by HMFG antigens. This approach is only valid if the rest of the organ against which the antibodies are directed is nonessential to the organism as a whole. Obviously this will not be the case for either brain or kidney; however, breast and prostate, among the sites of common carcinomas, fit the premise. In this study, non-breast carcinomas used as controls grew undeterred, as unaffected bystanders, under large cocktail doses capable of inducing very significant arrest of growth in breast tumors. Remarkably, neither non-breast tumor cell lodgment nor rate of tumor growth were altered by the anti-breast epithelial cocktail injection, even after 8 weeks of injections. Undoubtedly, breast specificity of the present cocktail is proven by these clear cut results. Results such as these generate the expectation that in future clinical trials the cross-reactivity with non-breast tissues could be either reduced or absent. Further support to the lack of toxic effects of the cocktail is shown in this study, where weight curves for the mice in the experiment of “passive immunization” that received the cocktail for 8 weeks and as the result of it did not grow any appreciable breast tumors, were similar to the weight curves of un.injected nu/nu mice of same age. Nevertheless, the reservation should be placed that the MoAbs were created against human antigens and could possibly cross-react at a higher degree with assorted human tissues in vivo, rather than with the host nu/nu mouse tissues. The final test will always come in either clinical trials or better, as a previous step, in the nu/nu mouse multihuman tumor panel that we have proposed (16), whereby many human tumors grown in nu/nu mice are used in vivo to detect binding of tagged MoAbs.

It can then be visualized that the two testing systems for antitumor action of MoAbs presented are: passive immunization against breast tumor grafting (a metastatic phenomenon simulation), and direct attack of already established breast tumors, validate the therapeutic value of the MoAbs used and provide information on the ability of the antibodies to affect tumor cell lodgment and tumor growth. It should also be pointed out that the total doses of MoAbs injected into the nu/nu mice in this study are only 1.5 to 2 times those administered to human subjects on a per kg basis for the treatment of solid tumors in clinical trials (20–23). Taking into consideration that the antibodies used were not conjugated to any other substance, it is conceivable that their effectiveness could be improved by conjugating them with cytotoxic drugs, vegetable toxins, and/or radioactive elements. Nevertheless, all these approaches in breast cancer should be based, as is shown, on the use of a mixture or cocktail of MoAbs and in the efforts to produce MoAbs with maximal specificity to breast epithelium.

### REFERENCES


MoAb THERAPY OF HUMAN BREAST CARCINOMAS


Experimental Immunotherapy of Human Breast Carcinomas Implanted in Nude Mice with a Mixture of Monoclonal Antibodies against Human Milk Fat Globule Components

Roberto L. Ceriani, Edward W. Blank and Jerry A. Peterson