Immunoscintigraphy of Human Mammary Carcinoma Xenografts Using Monoclonal Antibodies 12H12 and BM-2 Labeled with 99mTc and Radioiodine

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ABSTRACT

For immunoscintigraphic localization of human breast cancer two monoclonal antibodies (mabs) 12H12 (immunoglobulin G1) and BM-2 (immunoglobulin G3) were developed. The mabs, directed against two different epitopes on the mucin glycoprotein TAG-12, showed reactivity with 96% of all primary mammary carcinomas. The antibodies were labeled with either 125I or 131I. In addition, 12H12 was directly labeled with 99mTc according to the method of Schwarz and Steinstrasser (A. Schwarz and A. Steinstrasser, J. Nucl. Med., 28: 721, 1987). Biodistribution was measured in female nude mice bearing the human mammary carcinoma SF-15. Both radiiodinated mabs showed similar biodistribution with fast tumor uptake (8.5% injected dose/g at 6 h postinjection), which increased to 10-11% injected dose/g at 24 h and subsequently remained constant up to 120 h. 99mTc-Labeling of the mab 12H12 led to an enhanced tumor uptake of 10.5 and 14% Injected dose/g at 6 and 24 h postinjection, respectively, and to significantly accelerated blood clearance of radioactivity. Similar results were obtained with a second mammary tumor (AR-i), while an endometrial tumor (EK-3) showed a 3-fold lower accumulation of radioactivity and no difference in uptake of radiiodinated and 99mTc-labeled 12H12. Scintigraphic imaging of tumor-bearing nude mice with the 99mTc 12H12 at 24 h postinjection clearly demonstrated a diagnostic potential of the new mab for tumor localization and staging.

INTRODUCTION

Appropriate therapy for breast cancer depends upon reliable tumor detection and tumor staging. Since the presence of metastases in local lymph nodes is one of the major prognostic factors in the absence of distant metastases, large efforts have been made to better determine the extent of lymph node involvement by means of immunolymphoscintigraphy (1-5). However, the method seems to be limited by the specificity of the antibodies administered and the resolution of the equipment. Another important diagnostic problem is the detection of distant metastases. Radiolabeled monoclonal antibodies directed against tumor-associated antigens could answer specific questions in this field of clinical oncology. One well established indication for immunoscintigraphy, e.g., in colorectal carcinomas, is the localization of suspected tumor recurrences indicated by increasing tumor market levels (6).

A large number of antibodies against different epitopes of human neoplastic breast tissue have been investigated previously. Many of them were directed to carcinomaembryonic antigen (1, 2, 7), TAG-72 (8, 9), cell surface antigens like the mabs2 B6.2 (10-12), anti-MME (13) or RCC1 (4) and various others (3, 14). But the majority of the mabs used for breast cancer imaging like HMFG1 and HMFG2 (5, 15), SM-3 (16), DF3 (17), Mc5 (18), and MA5 (19) have been shown to recognize epitopes on mucin molecules (20). However, a highly specific and sensitive monoclonal antibody for immunoscintigraphy and radioimmunotherapy has not yet been found (21). This study was undertaken to evaluate whether the newly developed anti-mucin mabs 12H12 and BM-2 could improve tumor/tissue ratios and thus facilitate immunoscintigraphic detection and staging of breast cancer. For this purpose radioiodinated mabs were characterized with respect to their biodistribution in nude mice bearing human tumor xenografts. Two breast carcinoma lines differing in antigen expression level and localization of antigen-expressing cells (luminal, basal), as well as an endometrial cancer cell line were used. Additionally a kit for 99mTc-labeling of the mab 12H12 was used for biodistribution and imaging studies.

MATERIALS AND METHODS

mab

The mab 12H12 was raised against tumor cells from a human mammary carcinoma xenograft (22). mab 12H12 (Kd = 8.7 × 10^-9 M^-1) is of IgG1 subtype and reacts with carbohydrate side chains on the tumor-associated antigen TAG-12. The corresponding antigen was purified from T47D cells and effusion fluids by 12H12 affinity chromatography and gelfiltration. The high-molecular-weight glycoprotein, termed TAG-12, was shown to be a member of the heterogeneous mucin family (23). Purified antigen was used to develop a second generation of mucin-specific antibodies. Deglycosylated TAG-12 was used as immunogen to select mab BM-2 (formerly called 2E11). The mab is of IgG3 subtype and reacts with high affinity (Kd = 6.6 × 10^-10 M^-1) with multiple epitopes expressed on native and deglycosylated TAG-12 (24). mab BM-2 was found to react with synthetic peptides of the 20-amino acid tandem repeat area of the human Muc1 gene, its minimum epitope being the sequence A-P-D-T-R. Both mabs were produced in hollow-fiber bioreactors (Cellpharm II, Nunc, Wiesbaden, Germany) and purified to homogeneity by a combination of protein A-Sepharose 4 Fast Flow (Pharmacia, Freiburg, Germany) and fast protein liquid chromatography gel filtration by using Superose 6 (Pharmacia).

Antigen Expression

As previously shown, the high-molecular-mass mucin glycoprotein TAG-12 is expressed in more than 96% (n = 206) of primary tumors and in 99% (n = 107) of metastases of human breast cancer. However, the situation is different after transplantation to nude mice; only 1 of 7 tumor xenografts was still immunohistochemically TAG-12 positive after 15 passages in nude mice.

The antigen TAG-12 can be found in cytoplasm, cell membranes, and secretory components. Western immunoblotting of tumor homogenates with 12H12 shows heterogeneous bands only in the high-molecular-weight range of 400,000, while BM-2 shows additional bands in the range of M, 220,000 and 180,000 (23). Immunocytochemical analysis of tumor cells in bone marrow, effusions, and peripheral blood of patients with breast cancer (n = 289) are characterized by a homogeneous TAG-12 expression (25). Both mabs do not react with human skin, liver, pancreas, spleen, small intestine, bone marrow, thymus, prostate gland, lymphocytes, mesothelium, and other tissues. They show a slight reaction with apical secretory components of the normal breast gland and with fetal lung and kidney. The mabs BM-2 and 12H12 were used in a sandwich type enzyme immunoassay for TAG-12 analysis in sera of...
patients with breast and other cancer types. In breast cancer elevated levels were found in 32.4% (n = 225) of patients with no clinical evidence of disease and in 67% (n = 196) of patients with metastatic disease (26).

Xenograft Tumors and Cell Lines

The mammary carcinoma xenograft SF-15 (generous gift from Dr. H. P. Fortmeyer, Frankfurt, Germany) was cut into three 3x3x1-mm slices and was implanted s.c. in the anterior lateral thoracic wall (27). Tumor xenografts that grew to a size of more than 1 cm within 8 weeks were removed aseptically and peripheral, nonneocarcinomastic tissue was used for passaging. AR-1, a human breast carcinoma cell line recently established from a primary carcinoma in our laboratory, was transplanted from cell cultures. The cell line shows homogeneous mucin expression. EK-3, a primary human endometrial carcinoma cell line was cultured in our laboratory. It was additionally established as solid tumor xenograft on nude mice under the same conditions as reported for SF-15 tumor xenograft. In contrast to the commonly transplanted MX-1 and MCF-7 tumor cell lines, implantation of estrogen pellets was not necessary to enable growth of the three tumor xenografts.

T47D, a human breast carcinoma cell line (28) was used for the determination of the immunoreactive fraction of the mabs after radiiodination. All cell lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (GIBCO, Paisley, United Kingdom), 50 units/ml penicillin and 50 μg/ml streptomycin.

Immunohistochemical Examination of Tumor Xenografts

Paraffin-embedded tumor tissue was cut into 6-μm-thick sections. mabs 12H12 and BM-2 were derivatized with biotin N-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) and 0.5 μg mab in 500 μl PBS with 1% BSA (Boehringer Mannheim) was added to each tissue section and incubated for 2 h. After washing with PBS, 500 μl streptavidin-peroxidase (Dianova, Hamburg, Germany) 1/1000 in PBS with 1% BSA, were added to the tissue sections and incubated for 1 h at room temperature. Sections were then washed thoroughly with PBS and incubated with the substrate for 30 min. The substrates used were 3,3'-diaminobenzidine (Sigma, Deisenhofen, Germany), 50 μg dissolved in 45 ml PBS and 5 ml Tris buffer (pH 7.6) to which 50 μl H2O2 (30%) were added, and 3-amino-9-ethylcarbazole (Kem-En-Tec, Copenhagen, Denmark), 5 μg dissolved in 1 ml of 96% alcohol to which 9 ml distilled water and 10 μl of H2O2 (30%) were added.

Labeling of mabs

Radioiodination of mabs 12H12 and BM-2. Antibody (100 μg, 1 mg/ml 0.1 M phosphate buffer, pH 7.4, was placed in a glass tube coated with 5 μg of Iodo-Gen (Pierce, Rockford, IL). After the addition of 5.5 MBq of either 125I (740 MBq/μg; 7.4 GBq/ml) or 131I (carrier free; 3.7 GBq/ml) (Amersham Buchler, Braunschweig, Germany), the mixture was allowed to stand for 4 min (29) at room temperature. Unreacted 125I/131I was separated by a centrifuged column procedure using Bio-Gel P30 (Bio-Rad, München, Germany). The iodination yield was 90%, resulting in a specific activity of 55 kBq 125I/μg 12H12 and 44 kBq 131I/μg BM-2, respectively.

125I Labeling of mab 12H12. 125I labeling of the mab 12H2 was carried out as described by Schwarz and Steinstrasser (30), using a labeling kit provided by Hoechst (Frankfurt, Germany). Briefly, a vial containing 2.7 mg of the sodium salt of propane tetraphosphomc acid and 0.12 mg of SnCl2 was preloaded with an excess of corresponding unlabeled mab.

RESULTS

Immunoreactivity. The IF of the monoclonal antibodies 12H12 and BM-2 after radiiodination was determined by linear extrapolation to binding at the infinite antigen excess in a double-inverse plot according to the method of Lindmo et al. (31). The IF of mab 12H12 after labeling with 125I was 55% and the IF of mab BM-2 after labeling with 131I was 87%. The IF after 125I labeling of mab 12H12 was 45%. Unspecific binding amounted to 2–3%.

Immunohistochemistry. In immunohistochemical examination with the use of biotinylated mab 12H12, the SF-15 and AR-1 tumors showed an average staining of >80% of tumor cells. Wide areas showed homogeneous staining of all tumor cells beside areas with definite negative subpopulations, corresponding to a borderline type-I/II antigen pattern according to Mattes et al. (32). Using the biotinylated mab BM-2, the AR-1 tumor showed strong, homogeneous staining of all cells (Fig. 1) corresponding to type I antigen pattern,
Fig. 1. Immunohistochemical staining of mammary carcinoma xenograft AR with mab BM-2-biotin/streptavidin-peroxidase and 3-amino-9-ethylcarbazole as substrate. Staining corresponds to type I antigen pattern with homogeneous staining of all tumor cells. × 320.

while the SF-15 tumor showed type 2 antigen pattern (Fig. 2). With both biotinylated mabs the endometrial carcinoma EK-3 showed staining of luminal edges of tumor cells forming glandular structures (Fig. 3) typical of type III antigen pattern.

Biodistribution of mabs in SF-15-bearing Nude Mice. The biodistribution of mabs 12H12 and BM-2 in nude mice carrying the mammary carcinoma xenograft SF-15 is shown in Table 1. Biodistribution of 125I-12H12 and 131I-BM-2 mabs were quite similar, whereas 99mTc accumulation following 99mTc-12H12 administration differed significantly from radioiodine accumulation in blood, kidneys, and tumor. A probability of error of <0.001 was calculated for the lower level of 99mTc in blood and the higher renal uptake (2-tailed t test for comparing the mean values of two groups). Differences in the tumor uptake of 99mTc-labeled mab 12H12 and 125I-labeled mab 12H12, respectively, appeared to be less pronounced, but these differences also proved to be of high significance (P < 0.01) because at all time points in each animal the accumulation of 99mTc exceeded that of 125I (paired t test). Based on the biodistribution in SF-15-bearing nude mice other mammary carcinoma xenografts were evaluated at 48 h p.i. where highest tumor:tissue ratios were expected.

A similar distribution pattern was found at 48 h in AR-1-bearing mice (Table 2). However, difference in tumor accumulation of 99mTc-labeled mab 12H12 and 125I-labeled mab 12H12 was even higher than in SF-15-bearing mice. In contrast, no significant difference in tumor uptake was noted in EK-3 tumors (Table 3).

Tumor:tissue ratios of 99mTc-labeled mab 12H12 and radioiodinated mabs 12H12 and BM-2 at 48 h p.i. are shown in Table 4. 99mTc labeling of mab 12H12 resulted in significantly enhanced tumor:tissue ratios in SF-15 and AR-1-bearing mice compared to radioiodinated mab 12H12.

In EK-3 tumor-bearing mice, tumor uptake of 99mTc-labeled 12H12 was similar to that of 125I-labeled mab 12H12. Tumor:tissue ratios

Fig. 2. Immunohistochemical staining of mammary carcinoma xenograft SF-15 with mab 12H12-biotin/streptavidin-peroxidase and 3,3'-diaminobenzidine as substrate. Staining corresponds to type II antigen pattern with staining of more than 50% of the tumor cells. × 320.
Table 1 Biodistribution of $^{125}$I-labeled mab 12H12, $^{99m}$Tc-labeled mab 12H12, and $^{131}$I-labeled mab BM-2 in SF-15 tumor-bearing nude mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{99m}$Tc labeled 12H12</th>
<th>$^{125}$I labeled 12H12</th>
<th>$^{131}$I labeled BM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>24 h</td>
<td>48 h</td>
<td>120 h</td>
</tr>
<tr>
<td>Tumor</td>
<td>10.56 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.77 ± 2.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.63 ± 4.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood</td>
<td>17.39 ± 1.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.56 ± 0.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.38 ± 1.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>7.27 ± 1.00</td>
<td>3.90 ± 0.37</td>
<td>2.84 ± 0.29</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.70 ± 0.62</td>
<td>2.17 ± 0.44</td>
<td>1.39 ± 0.24</td>
</tr>
<tr>
<td>Kidneys</td>
<td>15.83 ± 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.21 ± 2.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.38 ± 1.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.73 ± 0.15</td>
<td>0.76 ± 0.12</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>Bone</td>
<td>1.77 ± 0.29</td>
<td>1.02 ± 0.08</td>
<td>0.60 ± 0.19</td>
</tr>
<tr>
<td>Lungs</td>
<td>5.97 ± 0.75</td>
<td>3.60 ± 0.42</td>
<td>2.23 ± 0.43</td>
</tr>
</tbody>
</table>

Differed only due to the enhanced blood clearance of the $^{99m}$Tc label. Tumor: tissue ratios of $^{125}$I-labeled mab 12H12 and $^{131}$I-labeled mab BM-2 were not significantly different in all tumor-bearing mice.

Radioimmunoimaging. Imaging with $^{99m}$Tc-labeled mab 12H12 in nude mice bearing SF-15, AR-1, and EK-3 carcinoma xenografts was performed 1 day after injection, and SF-15 and AR-1 tumor xenografts were clearly visualized with high contrast between tumor tissue and blood pool or liver (Fig. 4). Despite the 10-fold higher dose of mab used for radioimmunoimaging, biodistribution of SF-15 tumor-bearing animals was essentially the same compared to the 1-$\mu$g dose used for biodistribution studies. Tumor weights were 1090 mg (Fig. 4a) and 150 mg (Fig. 4b) in SF-15-bearing animals, and 460 mg (Fig. 4c) and 670 mg (Fig. 4d) in AR-bearing animals, corresponding to an absolute activity of 5.8% ID (Fig. 4a), 2.3% ID (Fig. 4b), 7.5% ID (Fig. 4c) and 8.2% ID (Fig. 4d) in the tumors. Despite its extensive central necrosis tumor (Fig. 4a) was easily detectable by immunoscintigraphy.

### DISCUSSION

*In vivo* distribution studies with monoclonal antibodies 12H12 and BM-2 in mice bearing different tumor xenografts were undertaken to evaluate whether the favorable *in vitro* characteristics of the mabs (high $K_a$, low cross-reactivity with other tissues, immunohistochemical staining of more than 96% of the primary mammary carcinoma) correlated with high tumor accumulation and significant tumor: tissue ratios *in vivo*. Inhibition or enhancement of $^{99m}$Tc-12H12 and $^{125}$I-12H12 binding in the biodistribution studies using 1-$\mu$g of mab could be excluded, because preliminary experiments showed no differences in biodistribution up to 20-$\mu$g of the mab administered. mabs 12H12 and BM-2 were shown to recognize different epitopes on the antigen TAG-12 and cross-competition was not observed in enzyme-linked immunosorbent assay and cell binding studies (data not shown).

Despite the 10-fold higher affinity of mab BM-2 to the antigen TAG-12 in vitro and the higher immunoreactive fraction compared to the mab 12H12, no increase in tumor accumulation was observed. This may be explained by the "binding site barrier" theory suggested by Weinstein *et al.* (33), stating that nonuniformity of the antibody

Table 2 Biodistribution of $^{125}$I-labeled mab 12H12, $^{99m}$Tc-labeled mab 12H12, and $^{131}$I-labeled BM-2 in AR-1 tumor-bearing nude mice, 48 h p.i.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{99m}$Tc</th>
<th>$^{125}$I</th>
<th>$^{131}$I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>20.65 ± 4.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.89 ± 1.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.21 ± 1.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood</td>
<td>7.61 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.45 ± 1.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.26 ± 1.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>3.69 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.1 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.16 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.71 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.39 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.10 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.66 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.34 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bones</td>
<td>1.12 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.14 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.35 ± 0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.55 ± 0.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.61 ± 0.69&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> % ID/g tissue ± 1 SD, all data are means of 5 animals.
<sup>b</sup> $^{99m}$Tc significantly different ($P < 0.01$) from $^{125}$I (paired t test).
<sup>c</sup> $^{99m}$Tc significantly different ($P < 0.001$) from $^{131}$I (1 t test, 2-tailed).
distribution will tend to be increased by a high affinity of binding or a high concentration of antigenic sites (34). An increase in binding affinity of the antibody from $10^6$ to $10^{10}$ was shown to have only a small effect on mab tumor uptake (35). Fujimori et al. (36) examined from their model, they could draw the conclusion that very high affinity ($>10^{10} M^{-1}$) prevented the antibodies from homogeneous tumor penetration. Recently, experimental evidence for a binding site barrier was provided by Juweid et al. (37) in guinea pigs bearing LiO
data for the degradation of $^{99m}$Tc- and WI-labeled neogalactosylated poorly. Immunohistochemical examination of the tumor xenografts showed a good correlation with the classification suggested by Mattes et al. (32), comparing the results obtained by immunoscintigraphy and biodistribution studies. In both breast cancer xenografts, showing borderline type-I/I! antigen pattern, a strong tumor accumulation was obtained, whereas the endometrial carcinoma EK-3 (type III) accumulated poorly. Immunohistochemistry of primary mammary tumors obtained, whereas the endometrial carcinoma EK-3 (type III) accumulated poorly. Immunohistochemistry of primary mammary tumors demonstrated that more than 75% showed type I or type II antigen pattern. In comparison, the study of Mattes et al. showed that the breast cancer specimen examined with pancarcinoma antibody from I2Hi2, it might be speculated that the different tumors catabolize of $^{99m}$Tc from the mabs due to transchelation to sulphydryl-containing labeling of IgG3 might be possible by the newly developed technique...
12H12 (45). Since the target antigen TAG-12 is also used as a tumor marker for breast cancer (26), it will be the goal of future studies to evaluate whether the 99mTc-labeled mab 12H12 could localize tumor recurrence indicated by increasing TAG-12 serum levels in breast cancer patients.

ACKNOWLEDGMENTS

We wish to thank our colleague Dr. B. Gückel for providing mammary carcinoma cell line AR and Dr. H. P. Fortmeyer (Frankfurt/Main, Germany) for kindly providing SF-15 mammary carcinoma xenograft. We also thank A. Eichler, M. Stegmüller, M. Stadler, and T. Regiert for technical help and assistance, and M. Accad (Houston, TX) and Dr. T. Brümmendorf (Tübingen, Germany) for critical reading of the manuscript.

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