Tumor Localization by Combinations of Monoclonal Antibodies in a New Human Colon Carcinoma Cell Line (LIM1899)


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ABSTRACT

One of the problems of in vitro diagnosis and therapy of tumors with monoclonal antibodies is their heterogeneity with respect to antigen expression, with some cells expressing no antigen and others being weakly or strongly positive. Selected mixtures of antibodies to different antigens are therefore likely to react with more cells than single antibodies and be more effective for imaging and therapy. With this in mind, we have examined a new human colon cancer cell line (LIM1899) which has a heterogenous expression of several cell surface molecules: by flow cytometry 38% were carcinoembryonic antigen positive; 64%, human milk fat globule positive, and 73%, CD46 positive; 87% of tumor cells bound a mixture of all three antibodies in vitro. Some blocking of the binding of anti-human milk fat globule antibody by the anti-CD46 antibody was noted. LIM1899 was established as a xenograft in nude mice and in vitro biodistribution studies performed using antibodies alone or in combination. Mixtures of antibodies clearly showed a higher percentage of injected dose of antibody in the tumor than did single antibodies: one antibody gave 10%; two together, 17 to 21%; and all three together gave 29% of the injected dose in the tumor. Tumor: blood ratios were also superior for combinations of antibodies, provided that low doses of the antibodies were used; at higher doses the effect was lost. The study demonstrates that combinations of antibodies are better than single antibodies for localization, provided that the dose used is carefully selected.

INTRODUCTION

The radioimmunodetection and immunotherapy of tumors using MoAbs requires maximal delivery of antibody to the tumor, with minimal retention or localization in blood or in other organs. Delivery of a single MoAb to tumors may be limited by antigen expression, with some antigens being expressed in low amounts by all cells, and there may also be heterogeneity in expression within the tumor (1, 2) with some cells lacking antigen altogether. In addition, similar tumors including metastases of the same tumor may differentially express different antigens (3, 4). Mixtures of MoAbs to different specificities may therefore have certain advantages over single MoAbs as reagents for the detection and therapy of tumors. (a) By using multiple MoAbs, more MoAb may localize in the tumor because several antigens can be targeted at one time, almost equivalent to increasing the antigen density on the tumor cells which has been shown to increase the amount of MoAb reaching the tumor in vivo (5, 6). (b) Tumors expressing different antigens may be targeted with a mixture of MoAbs, thus potentially increasing the number of tumors detected in different patients. In studies performed elsewhere, Durrant et al. (3) used a panel of five MoAbs to three colorectal carcinoma antigens to study the binding characteristics on colorectal tumors excised from patients; all the tumors reacted with at least one MoAb, 50% bound all the MoAbs, but 9% of the tumors bound to only one MoAb, clearly indicating that a mixture of MoAbs could increase tumor detection in patients compared with a single MoAb. In addition, several studies have indicated that cocktails consisting of either antibodies alone (7) or antibodies conjugated to drugs may be more effective in the therapy of tumor xenografts. Also, Munz et al. (8) showed enhanced tumor contrast in immunoscintigraphy using a mixture of radionabeled antibody F(ab')2 fragments to different antigens in tumor xenografts in mice. None of these studies indicated whether more of the injected dose was delivered to the tumors by mixtures of MoAbs. Tumor: blood ratios are crucial for radioimmunoimaging purposes (9), and if more of the injected dose of radiolabeled MoAb localizes in the tumor without a concomitant increase in radioactivity in the blood, tumor visualization should be improved. By contrast, mixtures of MoAbs to CEA were used in hamsters bearing human tumors, but no increase in tumor uptake was observed (10), possibly as the MoAbs were to the same antigen. Recently, in clinical studies, Orr et al. (11), aware of the problems of heterogeneity, have designed “individual specific” cocktails of antibodies based on fluorescence profiles obtained in vitro, but being a clinical study, were not able to correlate this with in vivo potency of the immunoconjugates. In this study we use a new colon carcinoma cell line which is heterogenous in antigen expression and can be considered similar to the type of heterogeneity commonly found in human tumors. The cell line (LIM1899) was recently established as a subcutaneous tumor xenograft in nude mice, and three different monoclonal antibodies were tested both individually and as a cocktail, in vitro and in vivo, for the interaction of the antibodies and the effect on the localization in the tumor, measured as the percentage of the injected dose and as tumor: blood ratios.

MATERIALS AND METHODS

Tumor Cells. The LIM1899 cell line was derived from a biopsy specimen of an invasive colon carcinoma which had been classified histologically as a moderately differentiated tumor. The culture was established using an explant culture method (12), and cells were passaged when confluent, using 0.1% trypsin-0.02% EDTA. Karyotyping of the tumor was performed at the time of the sixth passage (13). The tumor was established as a xenograft in nude mice by injecting 106 cells s.c. into nude mice; it could be passaged thereafter at 21-day intervals. Histological examination of early and late passages indicated no substantial differences.

Monoclonal Antibodies. The murine MoAbs used were 27.1 (IgG1), an anti-HMFG antigen which reacts with colon tumors (8), I-1 (IgG1), reactive with CEA present on colon carcinoma but not on normal tissue

Received 9/27/89; revised 5/3/90.

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1. To whom requests for reprints should be addressed.

2. The abbreviations used are: MoAb, monoclonal antibody; CEA, carcinoembryonic antigen; HMFG, human milk fat globule; FITC, fluorescein isothiocyanate; TCA, trichloroacetic acid.
Flow Cytometric Analysis of Binding of MoAbs to L1M1899 Tumor Cells. Binding of each monoclonal antibody and combinations of MoAbs was investigated by flow cytometry using FACSscan (Becton Dickinson, Mountain View, CA), using both direct and indirect assays. For the indirect assay, 2 x 10^6 cells were incubated separately with 2 μg of MoAb I-1, 27.1, E4.3, or anti-Ly-2.1 or with combinations of MoAbs: 27.1+I-1; 1-1+E4.3; 27.1+E4.3; and 27.1+I-1+E4.3. The incubation was for 1 h at 4°C followed by three washes with phosphate buffer containing 2% calf serum before measuring cell-bound fluorescence on the FACSscan. The direct assay was performed using MoAbs conjugated to FITC (18), where 2 x 10^6 L1M1899 cells were incubated with 2 μg each of the same mixture as the indirect assay for 1 h at 4°C. The mixtures were washed twice in buffer and resuspended to 0.5 ml for analysis using Consort 30 software on the FACSscan; a marker was set on the right of the fluorescence profile obtained for cells incubated with the nonreactive Ly-2.1 antibody.

Flow Cytometric Analysis of the Sequential Use of Mixtures of MoAbs on L1M1899 Cells. An assay was performed to investigate possible blocking of the binding of one MoAb by MoAbs already bound to the L1M1899 cells using a method similar to a sequential interference assay (19). Briefly, in the first round, 2 x 10^6 L1M1899 cells were incubated for 45 min at room temperature with 100 μg of unlabeled MoAb and unbound antibody aspirated after centrifugation. The cells were resuspended (without washing) and incubated with 100 μg of a second unlabeled MoAb. After incubation and removal of the second MoAb, cells were incubated for 45 min at room temperature with 1 μg of FITC-labeled MoAb. Finally, the cells were washed 3 times, and their fluorescence was monitored on FACSscan. The incubation medium contained 0.1% sodium azide to prevent internalization of MoAbs.

Radiolabeling and Immunoreactivity of MoAbs. MoAbs were radiolabeled with 125I or 131I (Amersham International Ltd., Amersham, United Kingdom) using Enzymobead reagent (BioRad Laboratories, Richmond, CA) according to the manufacturer’s procedure. The percentage of radioactivity bound to protein was assessed by precipitation with 10% TCA. Immunoreactivity of the radiolabeled preparations was assessed using a direct cell-binding immunosassay (20), with L1M1899 as target cells and thymus cells as negative control cells. Results were expressed as the percentage of radioactivity bound to cells at antigen excess. The errors are expressed as standard errors and are the result of several assays performed in triplicate with the same radiolabeled preparation. The specific activity of the radiolabeled preparation was between 1.0 and 2.5 mCi/mg, and the radioactivity of all preparations was less than 5% precipitable with TCA. The immunoreactive fractions of radiolabeled MoAbs reacting with L1M1899 cells were MoAb 27.1, 55.3 ± 3.5%; MoAb I-1, 77.1 ± 2.8%; MoAb 27.1+E4.3, 27.1 ± 2.7%; anti-Ly-2.1 (negative control), <5%. The percentage of radioactivity bound to thymus cells from mice expressing the Ly-2.1 antigen was less than 5% for the antitumor MoAbs and 68 ± 3.1% for the anti-Ly-2.1 MoAb. These radiolabeled antibodies were considered to be suitable for in vivo use.

Biodistribution of MoAbs and Combinations of MoAbs. L1M1899 cells (10^7 cells) were injected s.c. into the right flank of Swiss nude mice. Experiments were performed when the tumors had reached 0.5 x 0.5 cm, 4 to 6 wk post injection. Biodistribution of radiodinated MoAbs was studied using the paired label technique (21); 125I-labeled MoAb or a mixture of 125I-MoAbs was administered with 131I-anti-Ly-2.1, the negative control antibody. The mice were killed at 48 h post injection, and the following tissues were assayed for radioactivity: blood; tumor; spleen; kidney; liver; stomach; gut; heart; lung; muscle; and carcass. γ emissions from both 125I and 131I were counted, and correction was made for carry-over of 131I radioactivity into the 125I channel. The results were expressed as ratios of cpm/g of tissue to cpm/g of blood (tissue: blood ratio or T:B), and the percentage of injected cpm/g in the tissue was also calculated. Three different biodistribution experiments were performed as outlined in Table 1.

RESULTS

Characterization of Cell Line. Spindle-shaped cells were observed growing out of the tumor explants, and these cells were passaged when the culture became confluent. The heterogeneous morphology of the cells had remained constant for over 3 yr of continuous passage. Karyotype studies revealed 47 chromosomes with a small population of cells being hypertetraploid, and there was a single chromosomal abnormality involving chromosome 2. Tumor growth was evident 4 wk after s.c. injection of cultured cells. The histology of the xenografted tumor was similar to that of the original tumor. The tumor could be readily passaged in nude mice and could be reestablished in culture thereafter.

Flow Cytometric Analysis of MoAb Binding. The results of direct and indirect fluorescence assays were similar, shown by the fluorescence profiles for each MoAb and each combination of MoAbs (Fig. 1). A marker was set to the right of the fluorescence profile for L1M1899 with the nonreactive anti-Ly-2.1 antibody (Fig. 1A), and the percentage of cells with greater fluorescence values than this marker value was calculated. The mean percentages of the cells reacting were MoAb 27.1, 64 ± 8% (Fig. 1B); MoAb E4.3, 73 ± 3% (Fig. 1C); MoAb I-1, 28 ± 6% (Fig. 1D); MoAbs 27.1+I-1, 62 ± 4% (Fig. 1E); MoAbs 27.1+E4.3, 62 ± 7% (Fig. 1F); MoAbs I-1+E4.3, 79 ± 2% (Fig. 1G); MoAbs I-1+E4.3+27.1, 87 ± 2% (Fig. 1H). No increase in the number of cells with bound antibody was seen with combinations of MoAbs 27.1+I-1, or MoAbs 27.1+E4.3, and in the latter combination a decrease in the percentage of cells occurred, suggesting some interference in binding between the two MoAbs. Fluorescence profiles of cells with combinations of MoAbs including MoAb 27.1 (Fig. 1, E and F) more closely resembled the profile of cells with MoAb 27.1 alone (Fig. 1B) than the profiles of cells with the other two MoAbs (Fig. 1, C and D) which may indicate some blocking by MoAb 27.1. When a combination of all three MoAbs was added to L1M1899 cells, a higher percentage (87%) of cells showed a fluorescence value greater than cells binding to any single MoAb (Fig. 1H). Thus, in vivo, combinations of antibodies can clearly lead to more cells reacting with antibody, but not invariably so, and the findings depend on the antibody/antigen system.

Flow Cytometric Analysis of Interference between MoAbs. The results of the sequential interference assay are shown in Fig. 2. There was no blocking of the binding of MoAb 27.1 by MoAb I-1, but some blocking of the binding of MoAb 27.1 occurred by MoAb E4.3 (Fig. 2a). The binding of MoAb I-1 was not blocked by either MoAb E4.3 or 27.1 (Fig. 2c). The binding of E4.3 to L1M1899 was considerably reduced when MoAb 27.1 was included in the first or second round of the assay (Fig. 2b). About 50% blocking of binding of MoAb E4.3 occurred when L1M1899 cells had been incubated with saturat-

### Table 1 Dose (μg) schedules for in vivo experiments

<table>
<thead>
<tr>
<th>Experiment 1 with the following groups</th>
<th>Experiment 2 with the following groups</th>
<th>Experiment 3 with the following groups</th>
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<td>a b c d e</td>
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<td>125I-27.1</td>
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<td>1 1 1 1 1</td>
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<tr>
<td>125I-E4.3</td>
<td>10 10 10 10 10</td>
<td>1 1 1 1 1</td>
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<tr>
<td>125I-I-1</td>
<td>10 10 10 10 10</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>131I-Anti-Ly-2.1</td>
<td>10 20 20 20 30</td>
<td>1 3 2 2 1</td>
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* a to e, groups receiving the different doses indicated below.
TUMOR LOCALIZATION WITH COMBINATIONS OF MoAbs

Fig. 1. Fluorescence profiles of LIM1899 with MoAbs. Relative cell number is shown on the y axis and fluorescence on the x axis: A, anti-Ly-2.1 antibody (negative control); B, MoAb 27.1; C, MoAb E4.3; D, MoAb 1-1; E, MoAbs 27.1+1-1; F, MoAbs 27.1+E4.3; G, MoAbs I-I+E4.3; H, MoAbs I-I+27.1+E4.3.

Fig. 2. Sequential exposure to different MoAbs to measure blocking. Mean fluorescence is shown for each combination of antibodies. First and second round incubations were performed with saturating amounts of unlabeled antibody, and the third round incubations were with 1 μg of FITC-labeled antibody.

of two MoAbs (Fig. 3b), showing that more of the injected dose remained in the blood when three MoAbs were administered together compared with a combination consisting of two MoAbs.

In a second study using a higher dose, the mean percentages of injected dose/g and the mean tumor:blood ratios for mice receiving 10 μg of each MoAb are also shown (Fig. 3). Mice receiving combinations of MoAbs showed the same percentage of the injected dose/g localized in the tumor compared with mice receiving single MoAbs (Fig. 3a). Tumor:blood ratios for the combinations of individual MoAbs were slightly higher than those for single MoAbs (1.1 to 1.4 compared with 0.7 to 0.9), but the differences were not significant. It was apparent that, at this dose, combinations of antibodies did not show any improvement over single MoAbs and were worse than the results obtained for the 1-μg dose (Fig. 3).

For mice receiving 1 μg of each MoAb, the specificity of localization and percentages of the injected dose/g in different organs are shown in Fig. 4. The results for the nonreactive anti-Ly-2.1 MoAb were similar in all the groups of mice with no specific localization to tumor or to any tissue. The administration of a combination of three MoAbs (1 μg of each) caused no increase in the percentage of the injected dose/g in organs or tissues compared with mice receiving single MoAbs (Fig. 3a). However, the tumor:blood ratios were clearly superior to each antibody alone or even two antibodies.

In addition, tumor:blood ratios were higher in mice receiving combinations of MoAbs compared with single MoAbs (1-μg dose). However, the combination of all three MoAbs did not give a higher tumor:blood ratio compared with the combination of two MoAbs (Fig. 3b), showing that more of the injected dose remained in the blood when three MoAbs were administered together compared with a combination consisting of two MoAbs.
TUMOR LOCALIZATION WITH COMBINATIONS OF MoAbs

Fig. 3. Mean percentage of the injected dose/g (a) and mean tumor:blood ratio (b) for LIM1899 tumors excised from nude mice receiving either a single MoAb or combinations of MoAbs. The preparations were 1, MoAb E4.3; 2, MoAb 27.1; and 3, MoAb I-1. Injected doses were in a, 1 µg (C) and 10 µg (W); and in b, 1 µg (W) and 10 µg (III).

Fig. 4. Specificity of localization and mean percentage of the injected dose/g for each organ, a, mean ± SE of the percentage of the injected dose/g for each MoAb compared with the injected dose/g for anti-Ly-2.1 in blood and each organ 24 h after administration of 1 µg of each antibody into three groups of mice; values for anti-Ly-2.1 are a mean of values for all groups. b, mean ± SE of the percentage of the injected dose/g for blood, tumor, and all organs from groups of mice receiving 1 µg each of mixtures of MoAbs; combinations are 1+2, MoAbs E4.3 +27.1; 1+3, MoAbs E4.3 +I-1; 2+3, MoAbs I-1, 27.1; 1+2+3, MoAbs E4.3, I-1, and 27.1. Organs are BL, blood; TU, tumor; SP, spleen; KI, kidney; LI, liver; ST, stomach; GU, gut; HE, heart; LU, lung; MU, muscle; CA, carcass.

DISCUSSION

On the basis that tumors are heterogeneous and express different amounts of different antigens, it has been suggested that combinations of monoclonal antibodies, used either alone (7) or when conjugated to drugs, would be more effective for the therapy of tumor xenografts in nude mice than single antibodies. However, it is not clear whether the improvement is due to an increase in the amounts of antibody reaching the tumor, or because antibodies react with more cells within a heterogeneous tumor. In addition, improved tumor imaging with mixtures of radiolabeled F(ab')2 fragments has been observed, possibly (8) due to an increase in the amount of MoAb F(ab')2 in the tumor, as increasing doses of a single MoAb fragment did not result in image enhancement. Nonetheless, it is not altogether clear whether multiple antibodies do counter a real advantage in the use of MoAbs for therapy or imaging, and we have addressed this herein.

In this study it was clear that there was an increase in the amount of radioiodine found in the tumors of mice receiving mixtures of monoclonal antibodies compared with those receiving the same dose of a single antibody. Furthermore, the increase in tumor localization was dose dependent, at smaller doses (1 and 3 µg) an increase in the percentage of the injected dose/g localizing specifically in the tumor occurred; such an advantage was lost at higher doses. When 3 µg of a MoAb mixture were injected consisting of 1 µg each of three different bodies of the mice compared with mice receiving a combination of two MoAbs. The percentage of the injected dose/g occurring in all organs (except tumor) is similar if single MoAbs are compared with a combination of MoAbs (Fig. 4) and the combination of antibodies did not give higher nonspecific binding than individual antibodies.

The effects of increasing the dose of a single MoAb to the same level as the combinations of MoAbs was also examined (Fig. 5). In the tumors of mice receiving 3 µg of MoAb E4.3 there was a significant increase in the percentage of the injected dose/g compared with that in tumors obtained from mice receiving 1 µg of MoAb E4.3 (14.83% compared with 9.98; P < 0.001 by the Mann Whitney U test). However, the percentages of the injected dose localizing in the tumor did not show an increase when 30 µg of 125I-labeled MoAb E4.3 were administered compared with 10 µg of 125I-E4.3 (Fig. 5b). Increasing doses of radiolabeled MoAb led to decreased tumor:blood ratios due to a greater percentage of injected dose/g remaining in the blood (Fig. 5c). It was again clear that the nonspecific anti-Ly-2.1 antibody did not localize specifically in the tumor.
antigens are equally accessible, in vivo, an exposed surface is more accessible than surfaces adjacent to other cells, and cells deep inside a tumor are clearly not as accessible as those near the periphery. Finally, large doses of antibody may lead to a greater proportion binding to Fc receptors on different cells. Defining the reason for this dose effect may not be as important as the realization that increasing doses may not lead to a greater benefit, particularly for imaging studies where higher doses lead to a dramatic fall in the tumor:blood ratio, both by decreasing the value of T (% of injected dose in the tumor) and by increasing the absolute amount of radioactivity in the blood. Indeed, such was the case as in the tumor:blood ratio (Fig. 5c). Tumor:blood ratios for the single MoAb dose-response experiment decreased with increasing doses of antibody (Fig. 5c). This did not occur with the mixtures of MoAbs (Fig. 3b), where decreasing tumor:blood ratios suggest saturation of the tumor as they show more MoAb circulating in the blood rather than localized in the tumor. It is therefore important to select combinations of MoAbs and investigate dose schedules for use in therapy or imaging to obtain optimal delivery of MoAb and suitable tumor:blood ratios. Such conclusions underline the importance of careful phase I clinical imaging studies where dose escalation studies should begin at a sufficiently low dose to detect the problem described herein. However, for therapeutic studies, where the aim is to obtain a large amount of material in the tumor (irrespective of blood levels), then different parameters may well apply.

The in vitro data presented show that the tumor line used is not only heterogeneous in appearance but also in expression of the different antigens on different cells, and in the density of the same antigen on different cells (Fig. 1). Flow cytometry illustrates a wide spread of antigen expression, especially with respect to MoAbs 27.1 (anti-HMFG) (Fig. 1b) and MoAb I-1 (anti-CEA), which are expressed in low amounts on the cells, and two distinct populations of fluorescent cells occur with MoAb E4.3 (anti-HuLy-m5) (Fig. 1c and d). The combination of the three MoAbs showed a greater percentage of cell-bound fluorescence than any single MoAb, indicating that this combination would be expected to give improved localization to tumor in vivo. The sequential blocking experiment showed a more complex situation in vitro but not in vivo. MoAb 27.1 blocked approximately 50% of the binding of MoAb E4.3 (Fig. 2b), and the combination of MoAb 27.1 and E4.3 showed a greater percentage of the injected dose localizing in tumor (17.1 ± 3.13%) compared with either MoAb 27.1 (11.1 ± 0.66%) or MoAb E4.3 (10.85 ± 1.2%), when 1 µg of each MoAb was administered. This may be a reflection of the lower immunoreactive fraction of radiolabeled MoAb 27.1 (55.3%) compared with E4.3 (79.0%), or it may be that availability of antigens in vivo is different from that in vitro as discussed above.

Heterogeneity clearly poses a major problem when using monoclonal antibodies to both detect and treat tumors. With imaging, the variation in expression and density can lead to poor images unless the right antibody is used for that particular tumor. For therapy, far more serious consequences may result in that some cells may simply not be eradicated by the immunon conjugate as they receive no or little therapeutic material. Using carefully selected cocktails of antibodies would appear to be one answer to this difficult problem. In this light, it is of interest that similar conclusions were reached in several clinical studies (11) where in vivo coating of cells were approximately correlated with in vitro interaction of antibody.

REFERENCES


![Figure 5](image_url)
Tumor Localization by Combinations of Monoclonal Antibodies in a New Human Colon Carcinoma Cell Line (LIM1899)

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