Penetration and Binding of Radiolabeled Anti-Carcinoembryonic Antigen
Monoclonal Antibodies and Their Antibody Binding Fragments in
Human Colon Multicellular Tumor Spheroids

R. Sutherland, F. Buchegger, M. Schreyer, A. Vacca, and J-P. Mach

ABSTRACT

The binding and penetration of two \(^{125}\text{I}\)-labeled anti-carcinoembryonic antigen (CEA) monoclonal antibodies (MAb) and their F(ab')\(_2\) and Fab fragments were measured in multicellular spheroids of poorly (HT29) and moderately well differentiated (Col112) human colon adenocarcinomas which express different amounts of CEA. Spheroids cultured in vitro model tumor microenvironments where poor vascular supply may modulate antigen expression and accessibility. The two MAb studied, 202 and 35, were shown previously to react with different CEA epitopes and to have high affinities of 1.2 and 5.8 × 10\(^{10}\) M\(^{-1}\), respectively. MAb 202 has also been shown to cross-react with antibodies present on human granulocytes and normal epithelial cells from human lung and pancreas. Specific binding of intact MAb and fragments of both antibodies was demonstrated for both types of human colon carcinoma spheroids compared to mouse colon carcinoma (CL26) and mammary tumor (EMT6/Ro) spheroids. Total binding of MAb and fragments was greater (1.5- to 2.5-fold) after 4 h compared to 1 h of exposure; the amount of binding compared to control IgG1 was 5- to 30-fold greater after 1-h incubation and 15 to 200 times greater after 4 h. This binding was stable as demonstrated by short and long wash experiments at 37° and 4°C. The binding of F(ab')\(_2\) and Fab fragments of the anti-CEA MAb 35 to spheroids of human colon Col 12 was almost 2-fold greater than that of the intact MAb. However, for MAb 202, the binding of intact MAb and F(ab')\(_2\) was greater than that of Fab fragments. In addition the binding of both intact and F(ab')\(_2\) fragments of MAb 202 was greater than that obtained with MAb 35. Specific binding of both antibodies to HT29 spheroids, which express less CEA, was decreased for MAb and fragments of both 202 and 35. Autoradiography and immunoperoxidase experiments were performed to determine the penetration of MAb and fragments after incubation with intact spheroids. Comparisons were made with labeled MAb directly applied to frozen sections of spheroids. F(ab')\(_2\) and Fab fragments of both antibodies were bound at the surface of intact spheroids and penetrated to eight to ten cells, but the intact MAb were localized mainly at the spheroid surface and the outer one to three cell layers. There was much less binding at the surfaces of HT29 compared to Col112 spheroids. An enzyme immunoassay using MAb 35 and 202 demonstrated that Col112 spheroids produced about 8-fold more CEA/mg of cell protein than did monolayer cultures. CEA production in HT29 spheroids was less than Col112 by a factor of about 48. CEA expression as determined on frozen sections of spheroids was very heterogeneous and often associated with differentiated acinar structures. Despite the presence of abundant CEA, even the F(ab')\(_2\), and Fab fragments could not penetrate and bind significantly to most of these acini under these exposure conditions. These results demonstrate striking differences in binding of monoclonal antibodies and fragments to distinct CEA epitopes which might exhibit different biological expression.

INTRODUCTION

MAb against tumor-associated antigens provide the potential for improved diagnosis and assessment of disease status through radiolabeled imaging techniques (1) and \(\text{in vitro}\) immune assays (2). More tumor-specific directed therapy is also still an appealing possibility by using antibodies to deliver radioisotopes or other antitumor agents.

A major research effort is required to screen newly developed monoclonal antibodies to evaluate their stabilities, specificities, and other important properties which will determine their usefulness \(\text{in vivo}\). A prime consideration is the heterogeneity of reaction with subpopulations of tumor cells. Heterogeneous distribution of antibodies would be expected for most tumor types because of the well-known heterogeneity of tumor-associated antigen expression in humans (3-5). This cellular phenotypic heterogeneity may be determined by intrinsic properties of the cell population but may not be stable (6, 7). It is also probable that cellular antigenic expression will be modulated by cell-cell interactions and environments in tumor microregions. Heterogeneity associated with all these factors may be further compounded by abnormal microvascular function which may not only alter the cells in different microregions, but also the efficiency of delivery of the antibodies. Recently it has been possible to directly demonstrate such pathophysiological alterations of vascular function in different human malignant tumors (8, 9).

Evaluation of the effect of heterogeneity and poor vascularization on binding and distribution of monoclonal antibodies within human tumor microregions requires appropriate experimental model systems. In addition to \(\text{in vivo}\) xenografts of human tumors in immune-deficient mice, \(\text{in vitro}\) multicell tumor spheroids have been developed to simulate tumor microregions in three-dimensional growth which also facilitates cell-cell and cell-microenvironmental interactions (10-12). In the present research, spheroids of poorly and moderately well-differentiated human colon adenocarcinomas which express different levels of CEA have been used to determine the relative binding and distribution of two anti-CEA monoclonal antibodies and their F(ab')\(_2\) and Fab fragments. The two antibodies were chosen because of previous studies which showed that they bind to different CEA epitopes and indicated their promise for \(\text{in vivo}\) applications associated with differences in tumor/normal tissue distribution in nude mice and with apparent increased penetration of antibody fragments (13).

MATERIALS AND METHODS

Monoclonal Antibodies and Fragments. The derivation of hybridomas that secrete anti-CEA antibodies and their \(\text{in vitro}\) selections have been reviewed elsewhere (14). The abbreviations used are: MAb, monoclonal antibodies; CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.
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Described previously (14). Methods for purifying and testing of MAb 202 and 35, both of the IgG1 subclass, as well as for control IgG1 from myeloma cell line P3 × 63 Ag 8(Px) have also been described recently (15). Reciprocal binding inhibition tests showed that MAb 202 and 35 reacted with different epitopes. F(ab')2 fragments were obtained by 5% pepsine digestion at 37°C for 22 h (16) and Fab fragments by incubation for 6 h at 37°C with 3% papain (17) as described previously (13). Radiolabeling of intact MAb and their fragments with 125I was accomplished by the chloramine-T method. Free iodine was removed from the labeled proteins by dialysis or by Sephadex G-25 chromatography. The specific activity was 5 to 10 × 106 cpm per mg of MAb or fragments. MAb and fragments were tested for binding activity by incubation with 10 μg of CEA bound to cyanogen-bromide-activated Sepharose 4B (Pharmacia) overnight in 0.15 M PBS at 25°C.

Growth of Multicellular Spheroids. Human adenocarcinoma of lines Co112 and HT29 was used for these studies (18, 19). Co112 was in passages 36 to 41, and HT29 was in passages 170 to 175 during the period of this work. Cells in monolayer culture were used to initiate spheroid growth by resuspending with trypsin, treating with DME medium containing 10% fetal calf serum, and placing 3 × 105 cells in 10 ml of medium in sterile plastic non-tissue culture dishes (Nunc). After 4 days of aggregation and growth, the small spheroids (100- to 150-μm diameter) were placed in suspension culture in 100 ml of medium in spinner flasks at 150 rpm. The medium was replenished on alternate days during the first week and daily thereafter. Spheroids were removed periodically to maintain a relatively constant ratio of cells to medium during the 3- to 5-wk growth period. Human colon tumor spheroids used in the different experiments ranged from average diameters of 1100 to 1700 μm. In individual experiments similar (within 200 μm) diameters were used for comparative binding and penetration studies of MAb and fragments. Mouse CL26 colon tumor cells (20) and mouse EMT6/Ro mammary tumor cells (21) were also grown as spheroids (average diameter, 1100 μm) by this same method as described in detail previously (10, 12).

Incubation of Spheroids with MAb and Fragments. Five spheroids were placed in snap-cap plastic tubes (13 × 95 mm) in 1.0 ml final volume of DME medium plus 10% fetal calf serum containing the radiolabeled MAb or fragments. In the first experiment 10 spheroids were placed in 0.7 ml of medium, but this was changed in subsequent experiments to obtain better agitation. In all experiments, two to three tubes for each experimental condition were set up for simultaneous determinations of relative binding of MAb and fragments. The amount of radiolabeled MAb and fragments was 1 μg of protein (5 × 106 cpm per ml of 125I), except for the first study, where 5 μg of protein containing 20 × 106 cpm in 0.7 ml were used. In the first experiment the tubes were incubated at 37°C on a roller wheel at about 30 rpm. In the following experiments the tubes were placed at an angle in a test tube rack in a shaking 37°C water bath. Spheroids were washed twice in serum-free medium before beginning the incubations. The incubations were terminated after 1 or 4 h by aspiration of the medium and washing with isotope-free medium. A “short” wash consisted of aspiration of isotope-containing medium, resuspending the spheroids in 5 ml of medium, followed by aspiration and change of tubes for counting. The total wash time was no more than 10 min per tube. A “long” wash consisted of aspiration of isotope-containing medium, resuspending in medium for 60 min at 37°C or 4°C, and then aspiration, followed by one more cycle of resuspension, aspiration, and change of tubes for counting.

In 3 different experiments, binding of MAb and fragments of 35, 202, and Pd were more in early incubation with MAb 35 after 1 h (normalized to 1.0). In some of these experiments, additional tubes of spheroids were included to measure the relative binding of fragments after 1 h. Two additional experiments were performed to evaluate the relative binding of some of the MAb or fragments after 4-h incubation. The total number of separate experiments for each of the different conditions indicated in Table I. The mean ± SD of the total replicate measurements from all experiments was compared for statistical significance of Student's t test.

Immunoperoxidase Staining and Autoradiography. Spheroids were frozen in Optimal Cutting Temperature compound (Tissue Tech., Napperville, IL) in isopentane cooled by liquid nitrogen. Sections of 8 μm were prepared on glass slides. The distribution of CEA was assessed by immunoperoxidase staining of the frozen section using MAb anti-CEA and the avidin-biotin method (22). For autoradiography, either frozen sections or sections from spheroids fixed in 2.5% glutaraldehyde for 1 h were used. Fixed spheroids which had been incubated with radiolabeled MAb and fragments were embedded in glycol-methacrylate, sectioned at 2-μm thickness, dipped in Ilford nuclear emulsion type L4 (Ilford, Essex, United Kingdom), and developed from 1 wk to 4 mo later in Kodak D19 developer for 3 min and Kodak UNIFIX for 5 min. After washing and drying, these were counterstained with nuclear fast red or hematoxylin-eosin. Frozen sections of spheroids not previously incubated with radiolabeled MAb were used for the experiments on direct application of radiolabeled MAb (Fig. 7) and were processed for autoradiography as described above.

RESULTS

Kinetics and Stability of Binding of Intact MAb and Fragments. The two anti-CEA MAb 202 and 35 were selected for this study because their specificities, affinities, and binding properties in vitro, as well as in vivo tumor and normal tissue distributions in nude mice, are known (13, 14). Some relevant characteristics of these two MAb and their fragments are described here briefly. The affinities in PBS were 5.8 × 109 and 1.2 × 109 M⁻¹ for intact MAb 35 and 202, respectively, and 1.1 × 109 and 1.1 × 109 M⁻¹ for their respective Fab fragments. The maximum percentage of binding to an excess of CEA immobilized on Sepharose ranged between 85% and 55% for MAb and their fragments. MAb and Fab of 35 exhibited approximately the same reactivity (75 to 85%), while Fab of 35 was 65 to 70%, and Fab of 202 exhibited the lowest reactivity (50 to 55%). The binding of nonspecific antibody in these assays ranged from 0.3 to 2.1%. MAb 35 was the most specific, because it reacted only with CEA, whereas MAb 202 bound to a cross-reacting antigen with a molecular weight of 55,000 present on human granulocytes and normal epithelial cells of lung and pancreas as well as an antigen with a molecular weight of 95,000 also present on granulocytes (23). Because of these and other favorable characteristics, these two MAb have been selected, from among 32 hybrids that produced antibodies against CEA, as having the greatest potential for in vivo application.

The binding for different incubation times of MAb 202 and 35 and their fragments to multicellular tumor spheroids of human colon 112 adenocarcinoma cells is shown in Fig. 1. The amount of MAb and fragments bound to the Co112 spheroids after 4-h incubation at 37°C was always greater than after 1-h incubation. However, the relative amount of binding of the two MAb compared to their fragments was very similar after 4-h versus 1-h incubation. In general, the binding at 4 h was greater than that attained at 1 h by a factor of 1.5 to 2.5. The specificity of the binding is indicated by comparison with the amounts of binding of control (Px) MAb and fragments; this ranges between 5 and 30 times less (P < 0.025 to < 0.005) for 1-h incubation and 15 and 200 times less (P < 0.005 to < 0.001) for 4-h incubation, depending upon the particular MAb or fragment to which the Px binding is compared.

In experiments comparing the percentage of binding of the radiolabeled MAb and fragments to the spheroids in a 4-h incubation, binding ranged from 0.1 to 4.5 for MAb 35 and 0.7 to 18.5 for MAb 202. The fraction of the MAb and fragments which became bound was increased by decreasing the concentration of radiolabeled antibodies in the incubation medium as done in all experiments after the first (see “Materials and Methods”). Changing the spheroid concentration in the medium, or changing the agitation from roller tubes to tubes in a shaking water bath, did not substantially alter the results qualitatively or quantitatively, when expressed as relative amount of MAb or fragment bound per spheroid.
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Human Colon Co 112 Spheroids

- * 1 hour
- D = 4 hours

Fig. 1. Relative binding of 131I-labeled MAb, F(ab')2, and Fab fragments of anti-CEA 35 and 202 and control (Px) IgG to human colon Co 112 spheroids. The amount of binding based on cpm after 1 h or 4 h of exposure is normalized to the binding of MAb 35 of 1 h = 1.0. Numbers in parentheses, number of separate observations from the different experiments (see "Materials and Methods"). Each value is the average of 2 to 3 replicates for the number of separate experiments indicated. The standard deviation is indicated where 3 or more experiments were performed, and the range of the values is indicated when 2 experiments were performed.

Different washing procedures subsequent to the incubation periods were evaluated in order to determine the stability of the binding (Fig. 2). A short wash, of less than 10 min, and a long wash, of 1 h, at 37°C were also compared with 4°C. Details of the washing procedures are described in "Materials and Methods." The binding is normalized to the binding of MAb 35 = 1.0. Average of 2 experiments.

Fig. 2. Effect of different washing procedures on the stability of binding to human colon Co 112 spheroids of 131I-labeled MAb, F(ab')2, and Fab fragments of anti-CEA 35 and 202 after exposures of 1 and 4 h. Details of the washing procedures are described in "Materials and Methods." The binding is normalized to the binding of MAb 35 = 1.0. Average of 2 experiments.

Relative Binding of Intact MAb and Fragments. The data of Fig. 1 demonstrate that the binding of F(ab')2 (P < 0.1) and Fab (P < 0.05) fragments of the anti-CEA MAb 35 to spheroids of human colon Co 112 was greater than that of the intact MAb. In some, but not all, experiments, Fab binding was slightly greater than F(ab')2. On the other hand, the opposite results were obtained with 202; i.e., the binding of Fab was significantly less than that of the intact MAb (P < 0.005) and F(ab')2 (P < 0.01). The data of Fig. 1 also illustrate the significantly greater binding of 202 compared with 35, especially for intact MAb (P < 0.005) and the F(ab')2 (P < 0.01) fragment. This is relatively surprising, since the binding of all preparations of intact and fragmented MAb 35 and 202 to an excess of Sepharose-bound CEA was very similar, ranging from 55 to 85% (data not shown) and similar to results published previously (13).

Reactivity with Different Types of Spheroid Tumors. Human colon HT29 spheroids bound the MAb and fragments in a qualitatively similar manner to the Co 112 spheroids (Fig. 3), but the specific binding, especially for the Fab fragment, was quantitatively reduced as compared to the binding to Co 112 spheroids (Fig. 3 versus Fig. 1). This resulted from a decreased total binding to the HT29 spheroids as well as an increased nonspecific binding of the Px MAb and fragments. This lower specific binding, especially of MAb 35 and fragments, is likely due to the much lower CEA production of HT29 spheroids which was measured and found to be 20 to 80 times less (average, 47.5 ± 25.5 of 4 experiments) in 4 h compared to Co 112 spheroids.

The MAb and fragments against human CEA antigens did not cross-react significantly with their mouse colon tumor CL26 spheroids or with mouse mammary tumor EMT6 spheroids (Fig. 4, expanded scale, compared to previous figures). The binding to these spheroids in all cases was similar to that of Px. The supernatants of these spheroids contained no detectable CEA.
These spheroids contain acinar structures very similar to those in Col 12 spheroids used in these studies. Postlabeling differences could be seen in the labeling patterns for spheroids at 1 h, but there was little qualitative difference in the distribution compared to MAb and fragments, it is important to determine whether these acinar lumina in spheroids actually contained CEA as was reported previously for xenograft tumors of this cell line (13). Avidin-biotin immunoperoxidase staining of frozen sections of spheroids demonstrated heterogeneous distribution of CEA within the viable rim of cells and at the spheroid surface; however, CEA was predominantly in the acinar structures (Fig. 6). The acini are more frequently in the inner regions of the spheroids surrounding the necrotic centers, and CEA was present in greater amounts in these regions.

The amount of CEA was measured by enzyme immunoassay in extracts of Co112 spheroids, monolayers, as well as in culture medium supernatants (Table 1). The spheroids contained about 8 times more CEA/mg of cell protein present in the perchloric acid extracts. This was also reflected by a greater amount in the supernatants, approximately 4.5 times more released during 4 h.

To verify that radioiodinated MAb and fragments would bind to the CEA at these deeper regions within the spheroids if they had penetrated, frozen sections of spheroids were directly exposed on glass slides to the radioiodinated MAb and fragments. Fig. 7 is an autoradiograph which shows a very similar labeling pattern to the immunoperoxidase staining of Fig. 6. Thus, it appears that the radioiodinated labeling procedure or the presence of the $^{125}$I has not affected the ability of the MAb and fragments to bind to CEA. Consequently, the major limitation to binding of the MAb and fragments in these deeper regions of the intact spheroids appears to be the inability to penetrate. This is further substantiated by occasional observations of autoradiographs of fractured spheroids where penetration was facilitated and occasional labeling could be seen in the inner zone surrounding the necrotic centers.

**DISCUSSION**

These experiments have demonstrated several important points relevant to the potential for the use of radioiodinated monoclonal antibodies and their F(ab')$_2$ and Fab fragments for therapeutic and diagnostic purposes. The studies also illustrate how multicell spheroids of different tumor cells may be used to model tumor microregions to obtain insight into the types and extents of differences among antibodies and the reactions and distributions to be expected.

Two anti-CEA monoclonal antibodies, which had already been highly selected from 32 others based on favorable in vitro chemical and in vivo tumor and normal tissue studies in the Co112 xenograft tumor, were compared. Specific and stable binding was demonstrated for both intact MAb and fragments. However, the amount of binding and distribution differed significantly. There was much greater binding of 202 compared with 35. The 202 antibody, unlike 35, has been shown previously to bind to human granulocytes and normal epithelial cells of lung and pancreas. Thus, the greater binding of 202 might be due to the presence of NCA 55 in Co112 tumor cells (23), which can also be demonstrated on the spheroid surface using antibody and immunoperoxidase staining.

The binding of Fab 202 compared with MAb 202 was reduced by a factor of about 4 while, on the other hand, the relative binding of Fab 35 compared with MAb 35 was greater by a factor of about 2-fold. This opposite result was unexpected. An important factor which may partly explain this observation is the low affinity of Fab 202 (1.1 x 10$^8$) compared with Fab 35 (1.1 x 10$^9$). These fragments may also differ in cross-reactivity with other antigens such as NCA55 associated with differences in presentation and location of epitopes.

Autoradiographic studies showed the deeper penetration of
Fig. 5. Autoradiographic sections from Co112 spheroids exposed for 4 h to $^{35}$S-labeled anti-CEA antibodies and fragments. A viable rim of cells containing differentiated acinar structures surrounds a necrotic center. A, MAb 202 (× 300); B, MAb 202 (× 2000); C, MAb 35 (× 800); D, F(ab')2 202 (× 300); E, F(ab')2 202 (× 2000); F, Fab 202 (× 800); G, Fab 202 (× 300); H, Fab 202 (× 2000); I, Fab 35 (× 2000).
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Fig. 6. Avidin-biotin immunoperoxidase staining of sections of Col 12 spheroids, demonstrating heterogeneously distributed CEA within the viable rim of cells and predominantly in the acinar structures.

Table 1 Duplicate measurements from cell cultures in monolayers and from 10 spheroids

<table>
<thead>
<tr>
<th></th>
<th>ng CEA released in 4 h</th>
<th>ng CEA extracted from cells and spheroids</th>
<th>mg protein extracted from cells and spheroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co112 cells in monolayer</td>
<td>28.1 (15.04)</td>
<td>80.4 (43.0)</td>
<td>1.87</td>
</tr>
<tr>
<td>Co112 spheroids</td>
<td>181.3 (70.7)</td>
<td>735.0 (322.4)</td>
<td>2.28</td>
</tr>
</tbody>
</table>

* CEA enzyme immunoassay with MAb 35 and 202 as in Ref. 2 with modifications of incubation times and direct measurement in culture medium (sensitivity to 0.1 ng of CEA per ml of culture medium).

F(ab')2 and Fab fragments as compared to intact antibody. Much of the binding for both MAb and their fragments was at the spheroid surface, and significant penetration beyond about 8 to 10 cells in depth could not be achieved. Furthermore, CEA present in acinar lumen within this region and in deeper regions was rarely labeled by either MAb or direct fragments. Electron microscopy has shown that these acini contain tight junctional complexes between adjacent cells (24). It appears that these junctions present a significant barrier to the penetration of even the smaller Fab fragments of both antibodies.

Binding of MAb and fragments was specific for both human tumor spheroid cell types and did not occur for two mouse tumor spheroid cell types. However, significant differences were also observed between the two types of human colon tumor spheroids used in these experiments. Co112 spheroids express about 8 times more CEA on a per cellular protein basis than do the same cells grown as monolayer cultures. HT29 spheroids release into the medium about 20 to 80 times less CEA than Co112 spheroids. HT29 spheroids have no differentiated acinar structures compared to Co112 spheroids. The amount of specific binding to HT29 spheroids was less than that to Co112, and there was little surface labeling. These data indicate the possibility that significant differences in the accessibility and/or affinity of antigenic epitopes for MAb and fragments can occur in different tumors of similar general pathology but with differentiation differences.

It should be noted that the experimental conditions used to measure the uptake and binding of the MAb and fragments did not exactly match the growth conditions of the spheroids. Thus, the microenvironments within the spheroids may not have been the same as during growth and could possibly have affected the absolute amounts of binding. It is unlikely, however, that the practical experimental techniques necessary to measure the many variables could have significantly affected the results of the relative binding and distribution of the MAb and fragments.

Experiments on sections of spheroids directly exposed to anti-CEA antibody also demonstrated marked heterogeneity of distribution of CEA expression within the spheroids. It was
also shown that CEA is expressed (or retained) to a greater extent in association with acinar structures in the deeper regions of the viable rims of spheroids as compared to the necrotic centers which were only moderately labeled.

Previous in vivo experiments with Co112 xenografts in nude mice have indicated that fragments of the MAb 35 and 202 penetrate deeper into tumor nodules and are more favorable for in vivo use than intact antibodies for obtaining the greatest ratio of tumor to normal tissue (13). Our autoradiographic results largely confirmed the results of better tumor penetration obtained with fragments as compared to intact MAb. Furthermore, better tumor penetration of MAbs has also been demonstrated in nephrectomized mice (25). However, it does appear that penetration and binding to acinar structures were better in vivo, at least for the specific postinjection times examined in those experiments (48 h), than for the exposure times and conditions in these spheroid experiments (4 h). In addition to the longer exposure times in vivo, more labeling within acinar in vivo may occur if these structures are in a phase of formation during growth over the 48-h period of labeling similar to the seminacini which exhibited heavy labeling on the spheroid surface. Differences in the quality of junctional complexes in the acini in vivo or the presence of channels in the tumors in vivo through which MAb and fragments may penetrate in a manner similar to the penetration of fracture spheroids could also result in more labeling within acini in tumors than we observed in spheroids.

The multicell spheroid model of tumor microregions or micrometastases provides a useful supplement to other experimental in vitro and in vivo tumor systems to evaluate the heterogeneity of binding of antibodies and modulation of this by environments and changes in growth kinetics or differentiation of cell subpopulations (26). The penetration and distribution of antibodies when used for therapy, for example, labeled with isotopes of different energies, will be critical for determining therapeutic effectiveness in intervascular microregions of solid tumors. Experiments with spheroids to model these areas can provide important precise data on the geographical distribution of antibodies labeled with various isotopes versus cytotoxicity determined by isolating cell subpopulations from different regions of the spheroids. It may be possible to enhance the cytotoxicity of a radiolabeled antibody which exhibits heterogeneous distribution and limited penetration by multiple sequential administration of the antibody or combination therapy with other antibodies, moderate hyperthermia, radiation sensitizers, or other drugs.

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