Therapy, Department of Radiation Oncology, 5841 South Maryland Avenue, of page charges. This article must therefore be hereby marked advertisement in penetration distance of their emissions. The dose rates achieved et al. (9) have demonstrated this experimentally in xenografts of radiolabeled antibody therapy for micrometastatic disease. Most studies have calculated the dose to the tumor as a absorbed doses in the tumors and the biological effect of these studies of RIT because of their appropriate half-life and the measurements. Several ß-emitters have been used for most using autoradiography and microthermoluminescent dosimeter of radiolabeled Ab therapy (13). Ninety percent of the emitted ß energy from 131I is absorbed within a distance of 822 µm (14). 131I should therefore be an excellent radionuclide for studying the effectiveness of RIT in micrometastases less than 1 mm in diameter (15). This study assesses the dosimetry and biological effectiveness of 131I-labeled anti-CEA in LS174T human colon adenocarcinoma spheroids.

ABSTRACT
LS174T human colon adenocarcinoma multicell spheroids were used to study the radiobiological aspects of radioimmunotherapy. The spheroids were incubated in 131I-anti-carcinoembryonic antigen (B7) at an antibody concentration of 0.5 µg/ml and at 131I concentrations of 2.5 and 7.5 µCi/ml. After incubation times of 90 h, clonogenic cells per spheroid were reduced by 1600-fold and 23-fold at the high and low 131I concentrations, respectively. 131I Non-specific antibody (PX63) resulted in 2- and 1.2-fold reductions. Spheroid diameter was not significantly affected by therapy but histological examination revealed that there had been a significant reduction in the cell density, particularly near the spheroid surface. Using a theoretical model to estimate radiation dose, a radiation survival curve was constructed. The resulting curve was somewhat concave suggesting the presence of a resistant population of cells. It is likely that this observation is primarily due to the fact that the inner cells received a lower dose than the outer cells. A population of radiobiologically hypoxic cells in the inner portion of the spheroids may also have contributed to the decreasing slope of the curve as well as ongoing cell division leading to new cells which receive a lower radiation dose per cell cycle.

Because of the ability to estimate radiation dose for a given biological effect, these types of experiments may allow predictions of the efficacy of radiolabeled antibody therapy for micrometastatic disease.

INTRODUCTION
Tumor therapy using radiolabeled Abs5 to tumor-associated antigens is under active investigation by many groups. Studies in animals (1-4) have shown reductions in tumor volume and occasional disappearance of tumors. Early clinical trials in patients have been successful in reducing tumor size in a portion of the patients (5-8). One major problem in evaluating these studies has been the inability to accurately determine both the absorbed doses in the tumors and the biological effect of these doses. Most studies have calculated the dose to the tumor as a mean value throughout the tumor using conventional Medical Internal Radiation Dose techniques. Because of the heterogeneity of isotope deposition in tumors due to difficulties of Ab penetration and heterogeneity of antigen expression, the absorbed dose varies considerably throughout the tumor. Yorke et al. (9) have demonstrated this experimentally in xenografts using autoradiography and microthermoluminescent dosimeter measurements. Several ß-emitters have been used for most studies of RIT because of their appropriate half-life and the penetration distance of their emissions. The dose rates achieved in RIT using ß-emitters is generally thought to be quite low (<40 Gy/h for 90Y and <15 Gy/h for 131I) (10). Because the most actively proliferating tumor cells are likely to be near blood vessels, the dose rate may be much higher than is estimated using Medical Internal Radiation Dose techniques. On the other hand, hypoxic cells at a distance from feeding vessels, which are inherently resistant to radiation damage, may receive lower doses than estimated.

The multicell tumor spheroid contains a heterogeneous population of cells with microenvironments similar to in vivo tumors (11, 12). Because of this and the advantage of strict control of experimental conditions, the spheroid has proved to be a useful model to study the biological effectiveness of radiolabeled Ab therapy (13). Ninety percent of the emitted ß energy from 131I is absorbed within a distance of 822 µm (14). 131I should therefore be an excellent radionuclide for studying the effectiveness of RIT in micrometastases less than 1 mm in diameter (15). This study assesses the dosimetry and biological effectiveness of 131I-labeled anti-CEA in LS174T human colon adenocarcinoma spheroids.

MATERIALS AND METHODS

Cell Line. LS174T, a moderately well-differentiated human colon adenocarcinoma cell line, established in 1974, was obtained from Jeffrey Schom (NIH, Bethesda, MD) and used for these studies (16). LS174T expresses CEA and xenografts have been shown to have excellent uptake of anti-CEA Ab (17).

Spheroid Growth. Monolayer cultures were grown in Dulbecco's modified essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) and kept in a humidified atmosphere of 5% CO₂ and air at 37°C. Monolayer cells were trypsinized using 0.01% trypsin in 0.02% EDTA and a single cell suspension was layered over 2% Bactoagar (Difco, Detroit, MI). This was kept under the same growth conditions as the monolayer cells for 4 days. Small spheroids (80-100 µm diameter) formed under these conditions were then transferred to a spinner flask with fresh medium, gassed with 3% CO₂ and air, and spun at 110 rpm. Medium was supplemented every 2-3 days and the spinner rate was increased to 190 rpm when the spheroids reached 600-µm diameter. LS174T spheroids will grow to over 1.5-mm diameter. The diameter of 20-30 spheroids was measured under an inverted microscope in two perpendicular planes. The geometric mean of these two values was then calculated. Before experiments, the spheroids were passed through nylon mesh screens to obtain a population of uniform size.

Antibodies. B7, an IgG, murine MoAb to CEA was kindly given by J-P. Mach and F. Buchegger (University of Lausanne, Lausanne, Switzerland). Its affinity (Kₐ) in phosphate buffered saline is 1.6 x 10¹⁸/m. PX63, a mouse myeloma IgG₂ MoAb was used as a nonspecific isotype control.

Antibody Labeling. Iodination with 131I (New England Nuclear, Billerica, MA) was done using a modification of the chloramine T method (18) using 20 µCi iodine/µg MoAb and 10 µg chloramine T (Kodak, Rochester, NY)/µCi iodine. Labeled Ab was separated from free iodine by passage through a Sephadex PD-10 column (Pharmacia, Uppsala, Sweden). Trichloroacetic acid precipitation was performed before and after purification to determine the percentage of activity that was protein-bound. Specific activities from 5-15 µCi/µg MoAb were obtained and did not interfere significantly with immunoreactivity as...
determined by binding to CEA-Sepharose beads. Radiolabeled MoAb was filter sterilized (0.22 \mu m) prior to use.

Radiolabeled Antibody Therapy of Spheroids. LS174T spheroids of approximately 600-\mu m diameter were harvested, washed twice with medium not containing fetal calf serum, and placed in spinner flasks (300 spheroids/300 ml flask) with fresh medium and \(^{131}\)I-labeled MoAb at 37°C. MoAb concentration was 0.5 \mu g/ml and initial \(^{131}\)I concentration was either 2.5 or 7.5 \mu Ci/ml. Control flasks included untreated spheroids and \(^{131}\)I-PX63-treated spheroids. At intervals up to 90 h, spheroids were removed, washed four times in medium for 5 min per wash, sized and counted in a well-type gamma counter (Packard, Downers Grove, IL). Some spheroids were fixed for histological evaluation. The remaining spheroids were dissociated using 0.03\% trypsin in 0.02\% EDTA. It was necessary to then pass them through a 25-G needle to obtain a single cell suspension. The cells were then plated at varying dilutions to give 25-100 colonies per plate, incubated for 14 days, harvested, and stained with méthylène blue. Colonies of more than 50 cells were counted and plating efficiency determined, normalizing to the untreated controls. Corrections were made for multiplicity (see “Appendix”). Clonogenicity was expressed both as surviving fraction as compared to untreated control and as clonogenic cells per spheroid.

Dose Estimation. When intact anti-CEA is used, it has been shown previously that very little penetrates further than two cell layers in human colon adenocarcinoma spheroids and this has been confirmed in this cell line. We have developed a theoretical model for estimating dose at varying distances in from the surface of the spheroid, assuming that there is no significant penetration of Ab into the spheroid (15). The equation \( R(x,E_0) = AnkE_0\phi(x) cGy/s \) was used (14), where \( R \) is the absorbed dose rate, \( A \) is the activity in disintegrations/s, \( n \) is the number of particles of energy \( E_0 \)/disintegration, \( k \) is the average initial energy of emitted \( \beta \)-particles (MeV), \( \phi(x) \) is the specific absorbed fraction (g~'), and \( x \) is the distance from source (cm). The dose at any point in the spheroid was then estimated by integrating the dose rate from successive surface strips of activity of width \( r d\theta \) (in radians, \( r \) is the radius of sphere). Mean dose per cell was then estimated at different incubation times for the outer 200 \mu m of the spheroid which is the average thickness of the viable rim of cells. Total estimated doses included beta dose from bound \(^{131}\I\) labeled Ab, as well as \( \beta \) and \( \gamma \) doses from the \(^{131}\)I in the incubating medium. For comparison, the dose from bound antibody was also calculated assuming an even distribution of radionuclide in the spheroids using Loevinger's \( \beta \) particle point source function adapted for the special case of a sphere (20). A linear quadratic regression line was estimated for the natural logarithm of the surviving fraction as a function of dose. The standard error and the P value for the estimated coefficients are reported.

DNA Analysis. For studies of cell cycle distribution, dissociated spheroid cells were fixed in 70\% ethanol and allowed to decay. The cells were incubated in propidium iodide (10 \mu g/ml) and then treated with RNase (10 \mu g/ml) for 30 min to eliminate the binding of propidium iodide to RNA. The cells were then analyzed in a flow cytometer (Epics Profile; Coulter, Hialeah, FL). Data was analyzed using a cytology analysis program from the IBM-AT computer.

External Beam Spheroid Irradiation. Spheroids of 800-1000-\mu m diameter were placed in spinner flasks at a concentration of 50-60 per flask and gassed with 3\% CO_2/air. Forty-eight h later, the flasks were irradiated using a \(^{137}\)Cs Shepherd irradiator at 170 cGy/min. Control flasks were irradiated at 37°C. For full reoxygenation, flasks were irradiated at 37°C. The flasks were then spun through oil (4:1 dibutylphthalate:corn oil), frozen, and sectioned on a cryostat at a thickness of 5 \mu m. Slides were dipped in NTB-3 emulsion (Kodak, Rochester, NY) and developed using D-19 developer (Kodak). Sections were counterstained with hematoxylin & eosin. Bar, 30 \mu m. Original magnification, 32 x.

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\text{Fig. 1. Antibody binding versus incubation time from one experiment. Binding is expressed per unit spheroid surface area. }^{131}\text{I-labeled specific (B7) and nonspecific (PX63) antibody are shown.}
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\text{Fig. 2. Autoradiograph of LS174T spheroid incubated in }^{125}\text{I-B7 for 48 h. The spheroid was spun through oil (4:1 dibutylphthalate:corn oil), frozen, and sectioned on a cryostat at a thickness of 5 } \mu \text{m. Slides were dipped in NTB-3 emulsion (Kodak, Rochester, NY) and developed using D-19 developer (Kodak). Sections were counterstained with hematoxylin & eosin. Bar, 30 } \mu \text{m. Original magnification, 32 x.}
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RESULTS

Antibody Binding. LS174T spheroids bind \(^{131}\text{I-anti-CEA avidly. Binding increases over the first 72 h (Fig. 1). This increase is likely due to continuing new expression of antigen, spheroid growth, as well as some degree of slow penetration of MoAb into the spheroid. The finite rate of binding of Ab to antigen on cells is so rapid that this would not contribute significantly to the slow increase in binding (data not shown). Autoradiography after 48 h incubation in \(^{125}\text{I-B7 shows that penetration of intact MoAb into these spheroids is minimal (Fig. 2). The decrease in binding at 90 h is probably due to killing and loss of tumor cells that had bound the Ab and does not occur during incubation with }^{131}\text{I-labeled Ab. Binding is expressed in ng/mm}^2\text{ spheroid surface area because binding is primarily to the spheroid surface and this allows comparison between spheroids of varying diameter. Incubation of spheroid sections with B7 Ab reveal that there is CEA expression within the spheroids but the antigen is not easily accessible when the intact spheroids are incubated in Ab.}

Spheroid Morphology. Both treated and untreated spheroids continued to grow throughout the incubation time (Fig. 3A). Those treated with \(^{131}\text{I-B7 grew only slightly more slowly than controls. Total cells retrieved per spheroid decreased for }^{131}\text{I-B7 treated and control spheroids (Fig. 3B). Histological examination showed considerable vacuolization and necrosis in }^{131}\text{I-B7 treated spheroids which was most notable near the spheroid surface (Fig. 4).}

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Clonogenic Assay. Clonogenic cells per spheroid remained fairly constant for both control and 131I-PX63 treated spheroids (Fig. 5). For 131I-B7-treated spheroids, there was a three log reduction for the high dose (0.5 µg/ml and 7.5 µCi/ml) and over one log for the low dose (0.5 µg/ml and 2.5 µCi/ml). Surviving fraction versus incubation time is shown in Fig. 6 for the high dose of 131I. In contrast to the clonogenic cells per spheroid, there is less than a three log reduction in surviving fraction. This is presumably because more killed cells than viable cells are lost from the spheroids during the incubation time with 131I-B7 resulting in the cell population which is plated to determine clonogenicity being enriched with viable cells.

Dose Estimation. Assuming surface binding of labeled Ab, estimated mean dose per cell to the outer 200 µm of the spheroids is plotted against surviving fraction in Fig. 7. A linear quadratic fit was made to the data and is also plotted. The estimate for the linear term was -0.382 ± 0.037 (P < 0.001). The estimate for the quadratic term was 0.0050 ± 0.0016 (P = 0.006). If even distribution of 131I is assumed, the dose from bound 131I-B7 is increased by a factor of 1.3. Therefore, some degree of penetration of 131I-B7 into the spheroids would lead to a dose factor increase of less than 1.3. It should be noted that the points for the low 131I concentration fall within the data for the high concentration despite the lower dose rate for these points. For 131I-B7 treated spheroids after 90-h incubation at an 131I concentration of 7.5 µCi/ml, 91% of the total dose was from bound radiolabeled Ab and 9% was from 131I in the medium (7% from β emissions, 2% from γ rays).

DNA Analysis. Cell cycle distribution of dissociated spheroid
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Fig. 6. Surviving fraction versus incubation time for dissociated LS174T spheroids after incubation in I-131-MoAb at an I-131 concentration of 7.5 μCi/ml. Results were normalized to the plating efficiencies of untreated controls. Each data point represents one experiment.

Fig. 7. Radiation survival curve for LS174T spheroids treated with I-131-B7. Mean dose to outer rim is calculated as described in "Materials and Methods" and in Ref. 13. High and low are I-131 concentrations of 7.5 and 2.5 μCi/ml, respectively. Different symbols represent different experiments.

...cells at 90 h is shown in the DNA histogram in Fig. 8. The computer-estimated percentage of cells in S phase decreased from 25 to 15% and those in G0/G1 increased from 70 to 77% in I-131-B7-treated spheroids as compared to control. There was no change in G2/M. There was no evidence of G2 block and if LS174T follows the usual pattern of radiation sensitivity in early S phase, this shift may have had some influence on the survival data. However, the effect of this slight cell cycle redistribution should be of magnitude less than the total effect that was seen.

External Beam Spheroid Irradiation. Spheroids irradiated at 37°C showed a very sensitive radiation survival curve (Fig. 9) with virtually no shoulder. Survival curve parameters n, D0, and Dn, estimated from the high dose portion of the fully oxygenated curve at 4°C, were 3.2, 89 cGy, and 105 cGy, respectively. The terminal portion of the 37°C curve is less steep than the 4°C curve indicating the presence of a population of hypoxic cells. By comparing the terminal slopes of the air and nitrogen curves at 37°C which are parallel, the hypoxic fraction was calculated to be 10% (21). By comparing the 4°C air curve with the nitrogen curve, the oxygen enhancement ratio (ratio of nitrogen/air doses to give equal survival) was calculated to be 2.6.

DISCUSSION

This study demonstrates the effectiveness of radiolabeled MoAb therapy in small (<1 mm diameter) spheroids. Correlations between calculated radiation dose and biological effect were possible in this model. Because repair of sublethal damage may be ongoing during low dose rate radiation, the shoulder of the survival curve is continuously reconstructed. Therefore, tumors with little capability of repair of sublethal damage and a small shoulder on the radiation survival curve should show...
less of a dose rate effect than tumors with a large shoulder. As shown in Fig. 9, the LS174T cell line has a very small shoulder on the radiation survival curve. As the radiation survival curves for most colon cancer cell lines have a much larger shoulder, LS174T represents a best case scenario for RIT.

The radiation survival curve in Fig. 7 is concave rather than convex. The initial portion of the curve is quite steep but it becomes less steep as the dose increases, suggesting the presence of two or more populations of cells of differing radioresponsiveness. This is probably due to the fact that the cells near the spheroid surface received a much higher dose than those near the inner portion of the viable rim. As the dose was received in the same amount of time, the dose rate was also lower for the inner cells so that this resistant tail could also represent a dose-rate effect. However, despite the lower dose rates for spheroids incubated at the lower 131I concentration, the data still fell on the same curve as the high dose rate points. This may be because all repairable damage had been repaired at the higher dose rate and lowering it further did not allow any further repair (22). A resistant tail on the radiation survival curve often represents an hypoxic population of cells in the tumor. High dose rate external beam radiation studies indicate the presence of a 10% hypoxic fraction in LS174T spheroids (Fig. 9). (It should be noted that, because the x axes for Figs. 7 and 9 are different, they should not be directly compared.) These hypoxic cells may also be contributing to the shape of the curve, particularly since they are also likely receiving a low radiation dose given their location near the inner portion of the viable rim.

The maximum mean dose rate to the outer 200 μm from bound 131I-B7 was approximately 50 cGy/h and this occurred at 65 h. This represented a range of dose rates from over 100 cGy/h less than 20 μm from the spheroid surface to 30 cGy/h at 200 μm from the surface. It may be possible to obtain a more even dose distribution by using a much higher Ab concentration, Fab fragments instead of intact Ab, or by using an Ab of lower affinity (23, 24). Spheroids with fewer binding sites per cell or a large interstitial volume fraction may also result in better penetration of Ab. This would allow an estimation of the role of dose and dose rate distribution in producing the concave radiation-survival curve. Estimated doses in these experiments assumed that all cells were present from the beginning of treatment but cell division can occur at very low dose rates. Not only is dose per cell cycle important but successfully dividing cells are less likely to have sustained significant radiation damage. Therefore, continued cell growth may have contributed to the shape of the radiation survival curve.

Spheroid growth continued over the 90-h incubation period. A longer follow-up period of spheroid growth was not done. Despite an increase in spheroid diameter, the number of cells retrieved per spheroid was reduced in 131I-anti-CEA treated spheroids (Fig. 8). Histological examination and clonogenic assay demonstrated that very little of the volume increase was due to viable, clonogenic cells and, in fact, at 90 h there were fewer than 30 clonogenic cells per 131I-anti-CEA treated spheroids as opposed to over 3 × 10^4 clonogenic cells per spheroid in the controls. 131I-PX63 gave a very low radiation dose and clonogenic survival was close to control values (Fig. 5).

Because of the rapidly decreasing dose rate moving in from the spheroid surface, selective trypsinization (25) to remove successive cell layers from the spheroids would be useful in determining clonogenic survival at varying depths in the spheroid. Unfortunately, LS174T spheroids are very resistant to trypsinization and very few cells can be removed with trypsin alone. It is also necessary to pass the trypsinized spheroids through a 25-G needle to obtain a single cell suspension. Therefore it was not possible to use this technique in these experiments.

It has been suggested that cell cycle block at G1 or G2 may occur during continuous low dose rate radiation (26). This may influence the radiation effect on the cell population because of the accumulation of cells in sensitive or resistant phases of the cell cycle. In the experimental conditions for this study, there was a slight increase in the proportion of cells in G0/G1 suggesting the possibility of a block at G1. On the other hand, this may just be due to killing of cycling cells near the spheroid surface and sparing of noncycling cells further in.

The spheroid model represents an optimal situation for cell kill with radiolabeled Abs. There is no renal or hepatic clearance of Ab, dehalogenation is minimal, and there are no antigen-negative host cells to dilute the concentration of radionuclide. Prediction of dose distribution in a vascularized tumor in vivo is virtually impossible because of the unpredictable pattern of Ab binding. For these reasons, results from this model cannot be used to extrapolate directly into the in vivo situation. Of course, the main limitation to successful therapy is a limiting tumor/nontumor binding ratio as normal tissue toxicity is still the dose-limiting factor in radiolabeled Ab therapy. The future of RIT probably lies in a dual approach of minimizing normal tissue toxicity in one hand, and enhancing the tumor response on the other. Research should advance on these two fronts simultaneously.

**APPENDIX**

Correction for multiplicity was estimated by counting 100–200 cells in a hemocytometer. A_i is the number of clusters with i cells in it. The variable i was rarely greater than three but was evaluated up to i = 5. PE_M is the measured plating efficiency; PE is true plating efficiency; and PE_M is (total number of clusters) - (number of clusters that did not grow)/total number of cells plated:

\[
PE_M = \frac{\sum A_i}{\sum \sum A_i - \sum A_i PE}\]

This equation can then be solved for PE. This is most easily done using a short computer program.

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I\textsuperscript{131}I-ANTI-CEA THERAPY OF COLON CANCER SPHEROIDS


131I-Anticarcinoembryonic Antigen Therapy of LS174T Human Colon Adenocarcinoma Spheroids


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