Specific Killing of Human Melanoma Cells by $^{125}$I-Labeled 9.2.27 Monoclonal Antibody

Tore Lindmo, Epie Boven, James B. Mitchell, George Morstyn, and Paul A. Bunn, Jr.

NCI-Navy Medical Oncology, NCI-Navy Radiation Oncology Branches, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20814

ABSTRACT

The anti-melanoma antibody 9.2.27 localizes to melanoma cells when administered iv. to melanoma patients, but high doses of this antibody alone have no specific cytotoxic effect in vivo. To determine whether radiolabeled antibodies would exhibit specific antimalanoma cytotoxicity in vitro, cell survival curves were established for NCI-N892 human melanoma cells treated with $^{125}$I-labeled 9.2.27 monoclonal antibody. The binding capacity per cell was $5 \times 10^5$ molecules of 9.2.27 immunoglobulin G, and the association constant of binding was $10^{10}$ M$^{-1}$. Antibody preparations with specific radioactivities of 9–80 $\mu$Ci/µg were used. Colony-forming ability after in vitro exposure to $^{125}$I-9.2.27 was determined by a 1-h antibody incubation at saturating concentrations, washing, and cell freezing for various exposure durations. Colony survival was dose dependent, varying with the radioactivity per cell and the exposure time. The survival curves demonstrated no shoulder effect and had a 37% incremental survival dose of 0.5–0.9 $\times 10^6$ decays/cell.

Selective killing of melanoma cells was demonstrated in experiments where NCI-N417 lung cancer cells were mixed with the melanoma cells prior to antibody treatment. The NCI-N417 cells did not express the melanoma-associated antigen, were more sensitive to conventional external irradiation than were the melanoma cells, and could easily be distinguished from them by different growth morphology. In spite of a growth advantage for the melanoma cells in the cloning assay, the antigen-negative lung cancer cells selectively survived the treatment and were the only surviving cells after 15 days of exposure.

INTRODUCTION

The use of antibodies to obtain cell type-specific cytotoxicity for cancer therapy is an old concept which has become the subject of intense research effort after monoclonal antibodies became available (1–4). Clinical trials have demonstrated that monoclonal antibodies can be administered safely and can localize specifically to malignant cells, but administration of unlabelled antibodies alone rarely causes tumor regression. These considerations have intensified the interest in antibody conjugates. In recent years selective killing of cells expressing the relevant antigen has been demonstrated with monoclonal antibodies conjugated to toxins (5, 6).

To date radiolabeled monoclonal antibodies have been used primarily for tumor localization by means of external radioimaging in both animal tumor models and in patients (7–12). Preliminary clinical investigations of the potential therapeutic role of radiolabeled monoclonal antibodies (13–20) have shown definite but limited clinical utility. Two different views are prevalent concerning the choice of radiotherapeutic nuclides for coupling to monoclonal antibodies, and the aims are correspondingly different. With the use of $\beta$-emitters such as $^{131}$I and $^{90}$Y with radiations ranging up to one cm, the aim is to inactivate macroscopic solid tumors (13–15, 17). The dose at a certain point will be given by all the antibody accumulated within distances corresponding to the range of radiation. Thus in heterogeneous tumors where some tumor cells may not express the antibody-binding antigen, these cells will nevertheless be inactivated by the dose from surrounding antibody-binding cells.

A disadvantage of this scheme is, however, that the antibody bound to isolated antigen-positive cells will not be sufficient to inactivate such cells. For inactivation of isolated antibody-binding cells and micrometastases the radiation must be chosen to have a range comparable to the cell diameter and an efficient energy deposition per unit distance of absorption. This type of radiation would cause a highly localized effect and it might be possible to achieve specific killing of antibody-binding cells with little or no effect on neighboring antigen-negative, presumably normal cells.

Emitters of $\alpha$-particles or low energy electrons may be suitable nuclides for such applications of radiolabeled monoclonal antibodies. Warters et al. (21) have shown that $^{125}$I coupled to Con A caused dose-dependent inactivation of Con A-binding CHO cells, although the very limited range of the Auger electrons from $^{125}$I made the nuclide quite inefficient in delivering a lethal dose to the DNA of the nucleus. Preliminary experiments with antibodies labeled with $\alpha$-emitters have shown much more efficient cell inactivation (22, 23), but the specific inactivation of only antibody-binding cells has thus far not been demonstrated.

The aim of the present work was to investigate whether $^{125}$I-labeled monoclonal antibody against a melanoma-associated antigen would specifically inactivate antigen-positive melanoma cells without lethally damaging neighboring antigen-negative cells. After establishing dose-dependent cell survival curves for NCI-N892 melanoma cells exposed to $^{125}$I-labeled 9.2.27 anti-melanoma monoclonal antibody, we investigated the specificity.
of the effect by treating well-defined mixtures of antigen-positive and -negative cell types. We chose to freeze the cells after incubation with 125I-labeled antibody, thus making it possible to achieve different doses by varying the exposure time in the frozen state. This also eliminated the influence of cellular processes such as cell division and turnover of the antibody-antigen complex on the cell membrane that would otherwise complicate the determination of accumulated dose per cell (21, 24).

**MATERIALS AND METHODS**

**125I-Labeled Monoclonal Antibody 9.2.27.** The 9.2.27 is a murine monoclonal antibody of IgG2a class (25) directed against a Mr 250,000 melanoma-associated antigen (26). Purified antibody at a concentration of 1 mg/ml was obtained from Dr. A. C. Morgan (NCI-Frederick Cancer Research Facility, Frederick, MD). The antibody was labeled with 125I by the chloramine-T method (27), slightly modified to increase the specific radioactivity of the product (28). For each 10 µg of 9.2.27 we used 1 mCi of 125I (IMS 30; Amersham, Arlington Heights, IL) and 50 µl of chloramine-T (Eastman Kodak, Rochester, NY) at 25 µg/100 µl. The reaction was stopped by adding 20 mM sodium metabisulfite (Sigma Chemical Co., St. Louis, MO) in a volume equal to that of chloramine-T. By extending the reaction time from the normal 30 s to 2–10 min, monoclonal antibody with a specific radioactivity of 50–150 µCi/µg could be obtained. A Sephadex G-25 or G-75 column (Pharmacia, Stockholm, Sweden) was used to separate protein bound and free 125I after completion of the reaction. Prior to use the column was precipated with 1% BSA (Sigma) in PBS and subsequently washed with 0.2 M borate buffer (40–50 m) to remove unbound BSA. The same buffer was used to elute the column and the radiolabeled protein was collected via a sterile, disposable tubing system (Namic pressure monitoring line; North American Instruments Corp., Hudson Falls, NY). A shielded Geiger-Muller counter (Ludlum Measurements, Sweetwater, TX) positioned over the tubing close to the product vial was used to detect the passage of the radioactive protein fraction.

The protein concentration of the product was determined by the Bio-Rad (Bio-Rad Laboratories, Richmond, VA) microassay procedure, and its radioactivity was measured in a Capintec radioisotope calibrator (Capintech, Montvale, NJ) to calculate the specific radioactivity (µCi/µg). Precipitation in 6% trichloroacetic acid (Baker Chemical Co., Phillipsburg, NJ) was used to determine the fraction of protein bound 125I. Routinely about 95% of the radioactivity was precipitable with trichloroacetic acid. The immunoreactivity of the radiolabeled antibody was tested after each incubation with 125I-labeled antibody, thus making it possible to check that the number of colonies in each group was proportional to the number of cells seeded. Two independent plates of 10 wells each were included in each assay and the colony counts for treated cells were expressed relative to the mean plating efficiency of the two controls. On the average the two controls within each experiment differed by 18%, indicating an experimental error of ±9% in the clonogenic assay.

**External Irradiation.** Irradiation of NCI-N892 melanoma cells and NCI-N417 lung cancer cells was performed with a 6-MeV photon beam from a MeVatron VI linear accelerator at a dose rate of 2 Gy/min. The experimental procedure has been described previously (30). Both cell types were irradiated in suspension at room temperature and subsequently plated in 0.3% agarose as explained above.

Both cell types were also irradiated in the frozen state. Aliquots of cell suspensions of various concentrations up to 10 x 10⁶ cells/ml were frozen at −70°C in 1.5-ml vials (Nunc, Roskilde, Denmark) in RPMI 1640 containing 20% FCS and 7.5% DMSO (Merck, Darmstadt, Federal Republic of Germany). The cells were irradiated 1–10 days after freezing. A base layer of 2 ml RPMI 1640 containing 20% FCS and 7.5% DMSO (Merck, Darmstadt, Federal Republic of Germany) was placed in a well-humidified atmosphere of 5% CO₂ and 95% air, the number of colonies per dish was counted in an inverted microscope. In several cases three different cell concentrations were seeded for each group, thus making it possible to check that the number of colonies in each group was proportional to the number of cells seeded. Two independent plates of 10 wells each were included in each assay and the colony counts for treated cells were expressed relative to the mean plating efficiency of the two controls. On the average the two controls within each experiment differed by 18%, indicating an experimental error of ±9% in the clonogenic assay.

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Both cell lines were maintained in RPMI 1640 (Grand Island Biological Company, Grand Island, NY) supplemented with 10% heat-inactivated FCS (M.A. Bioproducts, Walkersville, MD). Cultures were grown without antibiotics at 37°C in a well-humidified atmosphere of 5% CO₂ in 95% air.

**Clonogenic Assays.** Single cell suspensions were washed and resuspended in RPMI 1640. The cell concentration was determined in a trypan blue assay and adjusted to 2 x 10⁶ to 1 x 10⁷ viable cells/ml, depending on the expected plating efficiency and surviving fraction. The clonogenic assay was set up in 35-mm Petri dishes (Costar or Falcon). Agarose (Seakem LE agarose; Marine Colloids, Inc., Rockland, ME) was made up at 3% in PBS, autoclaved, and diluted at 42°C to 0.3% in RPMI 1640 with 15% FCS, 1 µM L-glutamine (Grand Island Biological Co.) and penicillin-streptomycin (Grand Island Biological Co.) at concentrations of 100 units/ml and 100 µg/ml, respectively. A base layer of 2 ml agarose was added to each plate and allowed to settle before the top layer with cells was applied. For the top layer 4.5 ml of 0.3% agarose were added to 0.5 ml of cell suspension of the appropriate concentration, and 1 ml was seeded in each of four dishes. After 21 days of incubation at 37°C in a well-humidified atmosphere of 5% CO₂ and 95% air, the number of colonies per dish was counted in an inverted microscope. In several cases three different cell concentrations were seeded for each group, thus making it possible to check that the number of colonies in each group was proportional to the number of cells seeded. Two independent plates of 10 wells each were included in each assay and the colony counts for treated cells were expressed relative to the mean plating efficiency of the two controls. On the average the two controls within each experiment differed by 18%, indicating an experimental error of ±9% in the clonogenic assay.
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Distribution of cells in the frozen samples ($8 \times 10^8$ cells/ml) corresponded to an average distance between cells of about 50 μm, e.g., comparable to the average distance between leukocytes in peripheral blood. After varying intervals (1-45 days) the cells were thawed at 37°C, washed in 10 ml complete RPMI 1640, and plated in 0.3% agarose as explained above. About 75% of the radioactivity remained on the cells after washing. The radioactivity that was present on the cells upon plating was assumed to be so quickly diluted by cellular division and turnover of the antigen that it would represent a negligible additional dose.

Colony Typing. In experiments using mixtures of N892 and N417 cells, the cell type of each individual colony was identified by isolating individual clones from the agarose plates at the end of the incubation period of the clonogenic assay. Individual colonies were picked by a Pasteur pipet and transferred to 24-well culture plates (Costar) with 2 ml complete RPMI 1640 per well. After 1 week in culture, cell typing of individual colonies was done by microscopic inspection of growth morphology (Fig. 1), or the clones could be expanded by further growth in tissue culture flasks for subsequent analysis of antigen expression (Chart 1) or DNA content (Chart 2) by flow cytometric analysis.

Flow Cytometric Analysis. Cells were prepared for DNA measurement according to the procedure of Krishan (32) with slight modification. Suspensions of $10^8$ cells/ml were stained in $6.9 \times 10^{-5}$ M propidium iodide (Calbiochem, San Diego, CA) in $3.8 \times 10^{-3}$ M sodium citrate containing 0.01% Nonidet P-40 (Tergitol NP40; Sigma).

Indirect immunofluorescence analysis was performed by incubating single cell suspensions ($5 \times 10^5$ cells/0.2 ml) with the primary antibody at a saturating concentration of 15 μg/ml in PBS with 1% BSA. After 30 min incubation on ice, the cells were washed twice with PBS and incubated with 60 μl of the secondary, fluoresceinated antibody at a 1:50 dilution. For 9.2.27, fluorescein-conjugated goat anti-mouse IgG (H + L) (Becton Dickinson, Mountain View, CA), and for 534F8 (31), fluorescein-conjugated anti-mouse IgM (Becton Dickinson) were used as secondary antibodies. After 30 min incubation on ice with the secondary reagent, the cells were washed twice in PBS and subsequently analyzed with an EPICS V (Coulter Electronics, Hialeah, FL) flow cytometer. The laser line at 488 nm was used at 300 milliwatts for excitation of both propidium iodide and fluorescein. For DNA measurements the fluorescence was detected in the spectral range above 630 nm. Immunofluorescence was detected in the spectral interval from 515 to 560 nm, and a light scatter gate was set to register immunofluorescence only from intact cells.

RESULTS

Chart 3 shows survival curves for N892 and N417 cells after exposure to conventional external irradiation at room tempera-

Chart 3. Colony-forming ability after conventional external irradiation of NCI-N892 melanoma cells and NCI-N417 lung cancer cells at room temperature (A) and in the frozen state (−65°C) (B). The survival curve for NCI-N417 cells at room temperature has been published previously (30). Symbols represent independent experiments.

Chart 1. Flow cytometric immunofluorescence analysis of NCI-N892 melanoma cells (A, C) and NCI-N417 lung cancer cells (B, D) after staining with the monoclonal antibodies (MOAB) 9.2.27 (A, B) and 534F8 (C, D). The secondary antibody was fluorescein-conjugated anti-mouse IgG in case of 9.2.27 and anti-mouse IgM for 534F8. ——, control cells receiving only the secondary antibody.

Chart 2. Flow cytometric DNA histograms of NCI-N892 melanoma cells (A) and NCI-N417 lung cancer cells (B). Relative to diploid cells the DNA indices of N892 melanoma cells and N417 lung cancer cells were 2.0 and 1.3, respectively.

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ture (Chart 3A) and in the frozen state (Chart 3B). In both cases the N417 lung cancer cells were more sensitive than were the N892 melanoma cells. At room temperature the $D_0$ for the N892 cells was 1.6 Gy as opposed to 0.9 Gy for N417 cells. In the frozen state the values were 6.6 and 4.7 Gy, respectively, i.e., an increase in radioresistance by a factor of 4 for N892 cells and a factor of 5 for N417 cells. The dose response curves at room temperature had pronounced shoulders (extrapolation numbers, 2.4 and 5.6 for N892 and N417 cells, respectively), but this effect was markedly reduced in the frozen state (extrapolation numbers, 1.5 and 1.0, respectively).

Chart 4 shows dose-response curves based on two independent experiments for NCI-N892 cells exposed to $^{125}$I-labeled monoclonal antibody while in the frozen state. The antibody preparations used had specific radioactivities of 9 and 43 $\mu$Ci/ $\mu$g, respectively. The immunoreactive fractions were determined by a newly developed method (28) to be 45 and 37%, respectively. The bound radioactivities after washing were 2.2 and 2.7 dpm/cell for the antibody with weak specific radioactivity, compared to 10 and 11.2 dpm/cell for the one with medium specific radioactivity. This corresponds to about 0.11 $\mu$g of bound antibody per cell, which represents approximately 5 × 10^5 IgG molecules per cell, in agreement with the previously determined binding capacity of NCI-N892 cells for the 9.2.27 antibody (28).

Table 1 shows that within each experiment there was no significant difference in cell viability after thawing between different treatment groups. Freezing in 10% glycerol gave lower viability than did use of 7.5% DMSO, and glycerol was therefore used only in the first experiment. Inspection of the individual values underlying the mean values of Table 1 revealed no decrease in cell viability with increasing time in the frozen state for controls or for cells coated with unlabeled or radiolabeled antibody. Nor did the plating efficiency of control cells vary with time in the freezer.

From the survival curves of Chart 4A it is seen that incubation with unlabeled antibody had no effect on cell survival. The data for cells exposed to radioactive antibody were approximated by linear curves in the semilogarithmic plot. For determination of the $D_0$ the results in Chart 4A were replotted in Chart 4B as a function of accumulated disintegrations/cell, corrected for radioactive decay. The $D_0$ of 0.9 × 10^3 disintegrations/cell determined from the experiments with 11 dpm/cell seemed to be representative also for the experiments with 2.5 dpm/cell.

Specific killing of antibody-binding cells was studied in a model system consisting of mixtures of the NCI-N892 melanoma cells and NCI-N417 lung cancer cells. The cells were treated with $^{125}$I-labeled 9.2.27 monoclonal antibody with a specific radioactivity of 80 $\mu$Ci/ $\mu$g at saturating concentration. The immunoreactive fraction of the antibody was 63%, and the bound radioactivity per melanoma cell was about 24 dpm (Table 2). The viability of N417 cells after freezing/thawing was lower than for the melanoma cells, i.e., 57 versus 87%, and the N417 cells also had a lower plating efficiency, i.e., 4.6 versus 9.5% (Table 2). As in Table 1 the viability of treated cells was not significantly lower than for controls, and inspection of individual values for different time points showed no systematic variation.

Chart 5 shows the survival curves for four different cell populations relative to untreated controls of the same populations. Initially all three populations containing melanoma cells showed a steep decrease in cell survival. Too few cells were seeded in the pure melanoma cell population to determine the surviving fraction for exposure times longer than a few days. Cells plated on day 15 of exposure or later gave rise to no colonies in this group. From the number of seeded cells and the plating efficiency, the surviving fraction was calculated to be less than 6 × 10^-4. In analogy with Chart 4 a straight line was drawn based on the values determined for days 1 and 3, giving a $D_0$ of about 0.5 × 10^3 decays/cell.

The survival curves for N892 cell populations containing a 5 and 25% admixture of N417 cells demonstrated a biphasic relationship. After an initial decrease similar to that of the pure melanoma cell population, the survival curves leveled off. On this latter part of the curve the population containing 25% N417 cells...
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![Graph showing survival fraction vs. exposure time for NCI-N892 and NCI-N417 cells.](image)

Table 3

<table>
<thead>
<tr>
<th>Cell population</th>
<th>No. of colonies typed</th>
<th>% of N417</th>
</tr>
</thead>
<tbody>
<tr>
<td>N892</td>
<td>130</td>
<td>0/130</td>
</tr>
<tr>
<td>Untreated</td>
<td>130</td>
<td>0/130</td>
</tr>
<tr>
<td>Day 1</td>
<td>37</td>
<td>2/47</td>
</tr>
<tr>
<td>Day 3</td>
<td>16</td>
<td>3/26</td>
</tr>
<tr>
<td>Day 15</td>
<td>0</td>
<td>0/28</td>
</tr>
<tr>
<td>Day 28</td>
<td>0</td>
<td>16/18</td>
</tr>
<tr>
<td>Mixed*</td>
<td>37</td>
<td>2/47</td>
</tr>
<tr>
<td>N417</td>
<td>37</td>
<td>2/47</td>
</tr>
<tr>
<td>N417/total*</td>
<td>74</td>
<td>2/47</td>
</tr>
</tbody>
</table>

* Mixed colonies were assumed to consist of one colony of N417 lung cancer cells and one colony of N892 melanoma cells; i.e., they contributed twice to the total.

Table 4

<table>
<thead>
<tr>
<th>Type of radiation</th>
<th>Energy range (keV)</th>
<th>Dose contribution (%)</th>
<th>Range in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-rays and γ-rays</td>
<td>27-35</td>
<td>70</td>
<td>3 cm³</td>
</tr>
<tr>
<td>Medium energy electrons</td>
<td>22-35</td>
<td>15</td>
<td>15 μm</td>
</tr>
<tr>
<td>Low energy electrons</td>
<td>0.8-3.6</td>
<td>15</td>
<td>&lt;1 μm</td>
</tr>
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</table>

Chart 5. Colony-forming ability of pure NCI-N892 melanoma cells (•), pure NCI-N417 lung cancer cells (□), and the two mixed populations (△, A) exposed to membrane-bound ¹²⁵I-labeled 9.2.27 monoclonal antibody. The initial composition of the two mixed cell populations is indicated as the percentage of admixture of N417 cells to the N892 melanoma cells. The data are shown as a function of exposure time in the frozen state (−70°C) in 7.5% DMSO. In one determination of low cell survival so few cells were seeded that no colonies formed. The result therefore represents an upper limit for the surviving fraction at that particular exposure (△).

showed a 3–5 times higher survival than did the one with 5% N417 cells. Nonspecific binding of ¹²⁵I-labeled 9.2.27 to cells of the pure N417 population caused linearly decreasing cell survival at a Do of about 0.5 × 10⁶ decays/cell.

Individual colonies were picked for colony typing at the time of scoring of the clonogenic assays. Populations established from individual colonies were subjected to flow cytometric investigation of DNA content and of immunofluorescence after staining with the 9.2.27 and 534F8 antibodies. Invariably cell populations growing as floating aggregates, i.e., the pattern of N417 lung cancer cells, demonstrated the same DNA content as did the N417 parent population, and cells growing as attached monolayer had the same DNA content as the N892 melanoma parent cells. However, considerable variation in antigen expression between individual populations was found, i.e., in the quantitative binding of 534F8 to floating aggregate-type cell populations and 9.2.27 binding to monolayer-type cell populations. No case of floating aggregate-type cells positive for 9.2.27 or monolayer cells positive for 534F8 was found.

Table 3 summarizes the results obtained after colony cell typing of the population initially containing 25% N417 cells. The success rate in establishing growth of individually picked colonies was 55 of 60 for colonies from pure melanoma cell populations and 59 of 60 for colonies from pure N417 populations. Thus there was no difference in the efficiency of establishing populations from individual clones of N417 and N892 cells. However, of 130 colonies picked from the control population containing nominally 25% N417 cells, no population of floating aggregate morphology was established. Nevertheless all surviving cells of the treated population were of N417 type for exposure times of 15 days or longer.

DISCUSSION

The cytotoxic effect of radiolabeled antibodies bound to cell surface antigens depends upon the radiation quality of the chosen nuclide, the dose delivered to the critical component of the cell (i.e., DNA), and the response characteristics of the particular cell type. Specific killing of antibody-binding cells and selective survival of neighboring, antigen-negative, presumably normal cells can be achieved only with radiation of high energy deposition over a limited range comparable to the cell diameter. ¹²⁵I decays by electron capture, resulting in a cascade of low energy electrons. By electron capture one of the inner orbital electrons is absorbed by the nucleus, leading to an excited nucleus and an electron orbital vacancy. The vacancy is filled by electrons from higher orbitals and the excess energy of the process is emitted either as X-rays or as Auger electrons. The excited nucleus decays either by γ-emission or by the competing process of inner conversion, by which the excitation energy is used to eject an orbital electron. Tabulated frequencies and energies of the various processes (33–35) show that for ¹²⁵I the decay energy may be considered to consist of the three categories listed in Table 4. The component of interest is the electron emission with energy of 22–35 keV and with a range comparable to the cell diameter. The electrons of still lower energy (0.8–3.6 keV) will not contribute significantly to a DNA dose if originating from the cell membrane. The photon emission may in the present case be considered as weakly absorbed, penetrating radiation.

Based on these considerations we assume that the radiation...
from membrane-bound $^{125}$I will cause a localized effect, largely confined to those cells carrying the radiolabel.

The radioactivity per cell is proportional to the specific radioactivity of the antibody and to the number of binding sites per cell. In the present work we used antibody labeled to a specific activity of 9 to 80 $\mu$Ci/ug, although we have achieved as high as 150 $\mu$Ci/ug, corresponding to 10 atoms of $^{125}$I per antibody molecule (28). Generally there was a decrease in the immunoreactivity of the antibody with stronger labeling. A low immunoreactive fraction would cause problems for in vivo administration, representing a nonspecific dose of radioactivity, but in the present case the cells were labeled to saturation, and excess unbound and unreactive antibody was washed off. Scatchard analysis has shown that the NCI-N892 cells have about $5 \times 10^5$ binding sites per cell for the 9.2.27 antibody (28). This is in agreement with the saturation labeling data in this report using antibody with a specific radioactivity of 9–80 $\mu$Ci/ug and resulting in a radioactivity per cell of 2.2–24 dpm.

The dose-effect relationship shown in Chart 4 could be approximated by straight lines in the semilogarithmetic plot. The absence of a shoulder in the survival curves can be interpreted as caused by the absence of repairable sublethal damage due to an insignificant indirect effect of irradiation in the frozen state. In agreement with this Chart 3 shows that the shoulder effect on survival curves after conventional external irradiation was smaller in the frozen state than at room temperature (extrapolation numbers, 1.5 and 2.4, respectively, for N892 cells). The $D_0$ was the same for the antibody labeled to low (2.5 dpm/cell) or high specific radioactivity (24 dpm/cell) was about $5 \times 10^5$ decays/cell. In another experiment (Chart 5) the $D_0$ for the pure melanoma cell population exposed to radiolabeled antibody of high specific radioactivity (24 dpm/cell) was about $5 \times 10^5$ decays/cell. These values are high compared to the $D_0$ of 0.13 $\times 10^5$ decays/cell found by Warters et al. (21) in studies of CHO cells exposed to membrane-bound $^{125}$I at 4°C. The higher values in our case may be attributed to the increased radioresistance of the N892 cells in the frozen state.

An estimate of the effective DNA dose per decay of membrane-bound $^{125}$I can be made by relating to the results obtained for external irradiation. If the $D_0$ of 6.6 Gy for melanoma cells in the frozen state is set equivalent to the 0.5 $\times 10^5$ decays/cell determined from Chart 5, the resulting effective dose to the nucleus is $1.3 \times 10^5$ Gy/decay of membrane-bound $^{125}$I. This is in good agreement with the $1.8 \times 10^{-4}$ Gy/decay obtained similarly from the data by Warters et al. (21) and the value of $2.1 \times 10^{-4}$ Gy/decay obtained from their dosimetry calculations for CHO cells.

Admixture of the melanoma antigen-negative lung cancer cell line N417 was chosen as a model for cells that ideally should survive the treatment with radiolabeled anti-melanoma antibody. The pure N417 lung cancer cell population bound some radioactivity, although less than one-tenth of that of melanoma cells (Table 2). From other experiments we know that some nonspecific binding of the 9.2.27 antibody can occur, especially if the immunoreactive fraction is low (28). Flow cytometric studies have shown that nonspecific binding of antibody is most pronounced in dead cells (36). Since suspensions of N417 cells always contained about 50% dead cells (Table 2), a major fraction of the radioactivity in the pure N417 population was probably nonspecifically taken up by dead cells. The radioactivity per intact N417 cell would therefore be correspondingly less than 1.9 dpm/cell (Table 2), and the calculated $D_0$ for N417 cells of $0.5 \times 10^5$ decays/cell would be less if corrected for nonspecific binding to dead cells. This indicates that the N417 cells were at least as sensitive as the N892 melanoma cells to irradiation from $^{125}$I.

The shape of the survival curves for the mixed cell populations, shown in Chart 5, was as expected in the case of a resistant subpopulation. As demonstrated by the colony typing data (Table 3), the apparently resistant subpopulation consisted exclusively of N417 cells. Since their intrinsic radioresistance was equal to or higher than that of the melanoma cells (Chart 3), the comparatively high survival of N417 cells therefore must have been due to a lower radiation dose. Thus we have demonstrated that by using $^{125}$I-labeled anti-melanoma antibody, the radiation dose was selectively targeted to the antigen-positive cells, and neighboring antigen-negative cells experienced a higher survival.

The difference in survival between the two differently mixed cell populations was as expected from the difference in the percentage of N417 antigen-negative cells. However, the absolute survival level of the N417 cells was about 25 times lower than expected from the constitution of the mixtures and the assumption of ideal survival of antigen-negative cells. Results of colony typing in the control population containing 25% N417 cells (Table 3) indicated that the melanoma cells had a growth advantage over the lung cancer cells in the clonogenic assay that could explain this phenomenon. If corrected for this growth advantage of N892 cells in the clonogenic assay, the frequency of N417 colonies in the treated populations would have been higher, and totally selective survival of N417 cells would have been observed earlier than on day 15 of exposure.

The final slopes of the survival curves for mixed populations were steeper than for the pure N417 population. Thus the exposure times corresponding to the $D_0$ were 9 and 12.5 days for the 5 and 25% N417 populations, compared to 18 days for the pure N417 population. Considering that the N417 cells of mixed populations experienced 10 times higher bulk radioactivity than did the pure N417 population (Table 2), this indicated that the long range $\gamma$-emission from $^{125}$I contributed little to the observed effects. This conclusion was supported by results from a parallel study in which it was shown that free Na$^{125}$I added to the cell suspension prior to freezing caused only about one-sixth of the cytotoxicity of the same amount of membrane-bound radioactivity (37).

An important factor determining the efficiency of killing of the individual cell by membrane-bound radioactivity is what fraction of the dose actually is delivered to the DNA. Thus Warters et al. (21) have shown that $^{125}$I is much more efficient when incorporated into the DNA as [$^{125}$I]iododeoxyuridine compared to the effects obtained with $^{125}$I-labeled Con A bound to the cell membrane. Similarly Bloomer et al. (38) have studied the cell kill achieved by $^{125}$I-tamoxifen which binds to the estrogen receptor protein and is translocated to the nucleus. This strongly enhanced the effect of $^{125}$I, and Bloomer et al. found that $^{125}$I-tamoxifen was differentially cytotoxic in cells containing estrogen receptor.

Modulation of antigen expression by internalization of the antibody-receptor complex after binding could also lead to translocation of the radioisotope into the cytoplasm. The antigen on melanoma cells recognized by the 9.2.27 antibody does not modulate to any large extent (39), but other antigens show more...
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pronounced modulation, such as the T-cell antigen recognized by the T101 antibody (40, 41). Investigations have shown that after in vivo administration of 111In-T101 a significant fraction of the radiolabel was internalized due to antigen modulation (40, 42). This could potentiate the radiotherapeutic effect of 125I if coupled to the T101 antibody, since a larger fraction of the low energy electron emission would then reach the nucleus. Together with the specificity of cell kill demonstrated in the present report, this suggests that the possibility for in vivo therapeutic effect of 125I-labeled monoclonal antibodies should be evaluated in suitable systems.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. N. Carney for providing the NCI-N892 and NCI-N417 cell lines and to Dr. C. A. Morgan and Dr. F. Cuttitta for supplying the 9.2.27 and 534F8 monoclonal antibodies, respectively. Flow cytometric analyses performed by P. Jewett are gratefully acknowledged. This work was performed while two of the authors were visiting fellows at the National Cancer Institute. Helpful comments from Professor T. Brustad during the preparation of this manuscript are gratefully acknowledged.

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Fig. 1. Growth pattern of NCI-N892 melanoma cells (A) and NCI-N417 lung cancer cells (B). The photographs were taken through a ×10 objective on an inverted phase contrast microscope. Bar, 100 μm.
Specific Killing of Human Melanoma Cells by $^{125}\text{I}$-Labeled 9.2.27 Monoclonal Antibody


*Cancer Res* 1985;45:5080-5087.

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