Selective Growth Inhibition of Human Lung Cancer Cell Lines Bearing a Surface Glycoprotein gp160 by $^{125}$I-labeled Anti-gp160 Monoclonal Antibody

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ABSTRACT

Monoclonal antibody 5E8 which is specific for a M, 160,000 glycoprotein (gp160) on the surface of human lung cancer was radiolabeled with $^{125}$I. Radiolabeled 5E8 antibody is shown here to suppress the growth of gp160 positive human lung tumor cell lines in a dose-dependent fashion, but this same radiolabeled antibody does not alter the growth of gp160 negative lung tumor cell lines. Neither the unlabeled 5E8 nor a control radiolabeled monoclonal antibody has any effect upon the growth of gp160 positive tumors. The specificity of radiolabeled antibody mediated tumor killing is further demonstrated by the ability of unlabeled 5E8 to inhibit tumor killing by $^{125}$I-5E8. The efficiency with which the labeled tumor specific antibody suppressed tumor colony formation is enhanced by increasing the molar ratio of $^{125}$I to 5E8. This ratio could be increased to a level of two without affecting the capacity of the antibody to bind to the cell surface antigen. An attempt to increase the efficiency of tumor killing by the addition of a second antibody subsequent to incubation with $^{125}$I-5E8 was unsuccessful. These results indicate that $^{125}$I is a viable isotope and gp160 represents an appropriate target for radioimmunotherapy of human lung cancer.

INTRODUCTION

Monoclonal antibodies that bind to determinants on the surface of human tumors but not normal cells represent a great opportunity for improving cancer detection and therapy. With regard to therapy, most investigators have found that the antibodies per se have little or no effect upon tumor growth and that in order to be effective, the antibodies must be "armied" by covalent attachment of toxins, cytotoxic drugs, or by radiolabeling the antibodies (1). The feasibility of radiolabeling antibodies and the demonstration that such antibodies could be localized to a target organ was demonstrated 40 years ago (2). Subsequently, Pressman and his colleagues were also able to demonstrate the selective localization of radiolabeled anti-tumor antibodies to a tumor target (3). The recognition of tumor associated markers on human tumors such as the carcinoembryonic antigen (4) and the introduction of the hybridoma technology to produce monoclonal antibodies (5) have rekindled interest in radioimmuno therapy.

Recently, a new human tumor associated antigen was identified (6). This antigen is a gp160$^*$ found on the cell surface of many different histological types of human lung cancers and represents a potentially viable target for radioimmunotherapy. We have selected $^{125}$I as the radionuclide to test the feasibility of using our monoclonal anti-gp160 antibody for an antibody-mediated radiotherapy with gp160 positive human lung tumors. $^{125}$I emits many low-energy β particles (Auger electrons) per disintegration and is thought to be cytotoxic only when localized in close proximity to nuclear structures (7). The rationale for selecting this low-energy β emitter was based upon our knowledge that our monoclonal antibody is rapidly and efficiently internalized following its binding to the surface of the tumor cell. We anticipated, therefore, that $^{125}$I-labeled antibodies would be able to kill gp160 positive tumors by virtue of their ability to enter the cell and the use of $^{125}$I would reduce the nonspecific killing of bystander cells where the antibodies would neither bind nor enter the cell. We demonstrate here that up to two molecules of $^{125}$I can be coupled to our monoclonal antibody 5E8 without any noticeable decrease in the antibody's ability to bind to the tumor and that these radiolabeled antibodies are able to kill gp160 positive tumors selectively.

MATERIALS AND METHODS

Monoclonal Antibodies. The murine monoclonal antibodies, 5E8 (specific for the gp160 on human lung cancers) and 2C3 (specific for the hapten phthalate) were developed in our laboratory (6, 9). Both antibodies were isotyped as a γ1, heavy chain and κ light chain. Purification of these monoclonal antibodies was performed as described previously (6).

Radioiodination of Monoclonal Antibodies. Affinity purified 5E8 was radioiodinated by iodination with $^{125}$I using the iodogen method (10, 11). Briefly, 250 μl of three times concentrated borate buffer (pH 8.0) containing 50 μg of affinity purified monoclonal antibody and an appropriate volume of Na$^{125}$I ($^25$ mCi/ml in 0.1 N NaOH; ICN, Irvine, CA) were added to the iodogen tube to start the reaction. The iodogen tube was prepared by dissolving iodogen (Iodo-Gen; Pierce Chemical Co., Rockford, IL) in methylene chloride and adding 20 μg to the test tube followed by evaporation with nitrogen gas. After labeling, 100 μl of 1 M potassium iodide were added to displace $^{125}$I which was nonspecifically bound to the monoclonal antibody. $^{125}$I-labeled monoclonal antibody was then separated from free $^{125}$I on a Sephadex G-25 column (Pharmacia Fine Chemicals, Piscatway, NJ). This procedure was performed at room temperature. By prolonging the reaction time of iodogen (10 to 40 min) and/or varying the added volume of Na$^{125}$I (50 to 100 μl), six different preparations of $^{125}$I-5E8 were made. The molar ratios of $^{125}$I-labeled 5E8 and specific activities in these six preparations ranged from 0.355 to 2.580 and from 4.76 to 34.68 μCi/μg. The affinity purified 2C3 was also radioiodinated by the same method.

Immunoreactivity of $^{125}$I-labeled 5E8. The ability of each $^{125}$I-labeled 5E8 preparation to bind human gp160 positive lung cancer cell lines was tested by immunocytoadherence assay (8). Briefly, 40 μl of target cell suspension (2.5 × 10$^5$ cells/ml) and 50 μl of $^{125}$I-labeled 5E8 were incubated for 5 min at 4°C. After unbound $^{125}$I-labeled 5E8 was removed by repeating pelleting, 20 μl of a 1% suspension of sheep RBC conjugated with affinity purified rabbit anti-mouse immunoglobulin were added to the resuspended cells. The antibody pulsed tumor target cell and indicator sheep RBC were centrifuged for 3 min, resuspended, and examined under a microscope for the percentage of rosette forming cells. A rosette is defined as any target cell with five or more sheep RBC attached to its surface. The assay to determine the immunoreactive fraction of radiolabeled monoclonal antibody was performed according to the method of Lindmo el al. (12).

Human Lung Cancer Cell Lines. Human lung cancer cell lines used in this study were A549, an alveolar carcinoma (13), PC-1, a small cell carcinoma (14), A427, an adenocarcinoma (15), Calu-3, an adenocarcinoma (16), and QU-DB, a large cell carcinoma (17). All of these cell lines were maintained in RPMI 1640 medium supplemented with 10%
heat inactivated fetal calf serum. The cells were harvested by trypsinization (incubation in 0.05% trypsin and 0.04% EDTA in buffered saline), followed by washing three times with serum-free medium. Cell counts were done with trypan blue as an indicator of viability.

Colony Forming Assay. The clonogenic assay was carried out by the use of a modified method of Hamburger and Salmon (18).

Chronic Exposure of Lung Cancer Cell Lines to 125I-labeled Monoclonal Antibodies. Four hundred and ninety µl of RPMI 1640 containing 10% fetal calf serum and 0.24% agarose (FMC Corp., Rockland, ME) were pipetted into each well of a 24-well plate (Falcon: Becton-Dickinson, Cockeysville, MD) as a bottom layer, and 10 µl of various amounts of unlabeled or 125I-labeled monoclonal antibodies were added to the agar prior to gelling. Then the bottom layers were solidified at 4°C on a leveling plate for 30 min. The lung cancer cells were resuspended in RPMI 1640 containing 10% fetal calf serum and 0.22% agarose at a concentration of 2 x 10^5 cells/ml. Five hundred µl of the cell suspension were seeded over the bottom layer to yield a final concentration of 1 x 10^3 cells/well. After solidifying the top layers for 30 min at 4°C, the plates were incubated at 37°C in 5% CO₂ in a humidified atmosphere for 5 to 7 days. All plating was carried out in triplicate. Colonies containing five or more cells were counted using an inverted microscope. The percentage of inhibition of colony formation was calculated by the formula

\[
\% \text{ inhibition of colony formation} = \frac{C - E}{C} \times 100
\]

where \(C\) = the number of colonies in control group, which was not exposed to unlabeled or 125I-labeled antibodies, and \(E\) = the number of colonies in the experimental group, the group exposed to monoclonal antibodies.

Pulse of Lung Cancer Cell Lines with 125I-labeled Monoclonal Antibody. Five hundred µl of RPMI 1640 containing 10% fetal calf serum and 0.24% agarose were pipetted into each well as a bottom layer and allowed to solidify. One x 10^6 viable lung cancer cells were resuspended in 1 ml of DMEM containing various amounts of 125I-labeled 5E8 or 125I-labeled 2C3 and incubated for 5 min at 4°C. The unbound 125I-labeled antibody was removed by repeated pelleting of the cells. One x 10^6 viable cells pulsed with 125I-labeled antibody were resuspended in 500 µl of RPMI 1640 containing 10% fetal calf serum and 0.22% agarose and placed over the bottom layer. The colony forming assay was performed and the percentage of inhibition was determined as described above.

Cold Inhibition Test. Increasing amounts of unlabeled 5E8 were added to 5 µg of 125I-labeled 5E8 in 1 ml of DMEM to yield the mixtures in the following proportions: 1:0, 10:1, 2:1, 1:1, 1:2, and 1:10 as a protein ratio of 125I-labeled 5E8 to 125I-labeled 2C3. As a control, 5 µg of unlabeled 5E8 in 1 ml of DMEM were prepared. Five x 10^5 A549 cells were incubated in these mixtures and incubated for 5 min at 4°C. The unbound 125I-labeled antibody was removed by repeated pelleting of the cells. One x 10^5 viable cells were washed three times with serum-free medium. Cell pellets were harvested by trypsini-heat inactivated fetal calf serum. The cells were harvested by trypsinization (incubation in 0.05% trypsin and 0.04% EDTA in buffered saline), followed by washing three times with serum-free medium. Cell counts were done with trypan blue as an indicator of viability.

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RESULTS

Reactivity of 5E8 with Human Lung Cancer Cell Lines Determined by Immunocytotoadherence Assay. The presence of the tumor associated antigen gp160 on the surface of five different human lung tumor cell lines was examined by an immuno cytotoadherence assay using unlabeled 5E8 antibody. In this assay, the binding of 5E8 to cancer cell lines is detected by rabbit anti mouse immunoglobulin conjugated sheep RBC. Three different human lung tumor cell lines, i.e., A549, Calu-3, and A427 were shown to express gp160 on their cell surface (Table 1). Two other human lung tumor cell lines, i.e., PC-1 and QU-DB were determined to be negative for gp160 (Table 1). Of the three positive cell lines, the A549 cell line had the highest percentage of gp160 positive cells (85%).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>% rosette forming cells*</th>
<th>gp160 on the cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>Alveolar</td>
<td>85.1 ± 6.0</td>
<td>Positive</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Adeno</td>
<td>68.9 ± 3.1</td>
<td>Positive</td>
</tr>
<tr>
<td>A427</td>
<td>Adeno</td>
<td>50.0 ± 6.7</td>
<td>Positive</td>
</tr>
<tr>
<td>PC-1</td>
<td>Small cell</td>
<td>2.2 ± 2.6</td>
<td>Negative</td>
</tr>
<tr>
<td>QU-DB</td>
<td>Large cell</td>
<td>1.9 ± 1.3</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Percentage of tumor cells that bind five or more anti-mouse immunoglobulin coupled RBC following a pulse with the monoclonal anti-gp160 antibody.

Effect of Selective Inhibition of Growth of gploO Positive Tumors Mediated by 125I-labeled Monoclonal Antibody. When tumor cells were incubated briefly with 125I-labeled 5E8 and the unbound antibody was removed by repeated washings of the antibody-pulsed tumor cells, it was possible to show that gp160 on the surface of A549 cells when the ratio of 125I to antibody was 1:7:1 or less. At this level of iodination, we determined that the fraction of the labeled antibody capable of binding to gp160 was 0.933. However, at a higher ratio (2.5:1) there was a noticeable change in the binding of the labeled antibody to the tumor cells.

Selective Inhibition of Growth of gp160 Positive Tumors Mediated by 125I-labeled Monoclonal Antibody. When tumor cells were incubated briefly with 125I-labeled 5E8 and the unbound antibody was removed by repeated washings of the antibody-pulsed tumor cells, it was possible to show that gp160 positive but not gp160 negative tumors were killed (Fig. 2). This selective killing was dose dependent but the radiolabeled antitumor antibody did not inhibit all of the tumor colonies. At the highest dose tested, i.e., 1 µg of antibody/1 x 10^6 tumor cells, 75% of the A549 colonies were inhibited and only about one-half of the other two gp160 positive tumor colonies were inhibited.

This protocol with the radiolabeled antitumor antibody was repeated and the effects of the tumor specific antibody on tumor growth were compared to the effects of a control antibody that was iodinated to the same specific activity. The results presented in Fig. 3 indicate that the 125I-labeled 5E8 but not 125I-labeled 2C3 inhibited the colony formation of the three gp160 positive human lung tumor cell lines.

Effect of Chronic Exposure to 125I-labeled Monoclonal Antibodies on Tumor Growth. A remote possibility that would explain the failure of the 125I-labeled 5E8 to inhibit the growth...
of gp160 negative tumors was that these tumors were relatively radioresistant. In order to rule out this possibility, the radioiodinated monoclonal antibodies were incubated with gp160 positive (A549) and gp160 negative (QU-DB) tumor cells with increasing amounts of $^{125}$I-labeled 5E8 antibody or a similarly labeled control monoclonal antibody 2C3. In contrast to the previous protocol, where the unbound antibodies were removed prior to culture, here the antibodies were present for the entire culture period. After 7 days in culture with the radiolabeled antibodies, the number of tumor colonies was determined, and the percentage of inhibition of colony formation was calculated as indicated in "Materials and Methods." As illustrated in Fig. 4, the growth of the A549 cells was inhibited in a dose-dependent fashion by either $^{125}$I-labeled 5E8 or the control $^{125}$I-labeled 2C3 antibody. It was also determined that prolonged exposure of the gp160 negative tumor cells QU-DB to $^{125}$I-labeled 5E8 suppressed tumor growth in a dose-dependent fashion (Fig. 4). It was assumed that this nonspecific tumor suppression was due to the radioactivity from the labeled antibodies that were present throughout the culture period. This assumption was confirmed by the finding that neither 5E8 nor 2C3 antibodies without I25I had any effect upon the growth of the A549 tumor cells (data not shown). We conclude from the data presented in Fig. 4 that there is sufficient radioactivity associated with 1 $\mu$g of either $^{125}$I-labeled 5E8 or $^{125}$I-labeled 2C3 antibodies to kill either the gp160 positive A549 cells or the control QU-DB cells provided that these labeled antibodies are brought into close proximity of the tumor cells and remain associated with the cells for the duration of the culture period. These results rule out the possibility that the gp160 negative tumor cells are radioresistant.

Effect of Increasing Level of Iodination on Ability of Antibody to Kill Tumor Cells. An attempt was now made to determine if by increasing the molar ratio of $^{125}$I to 5E8 antibody protein one could enhance the ability of the antibody to inhibit tumor colony formation. As indicated in Fig. 5, a 7-fold increase in this ratio (from 0.355 to 2.58) did not alter the percentage of colonies inhibited but did increase the efficiency of antibody
The ability to successfully conjugate a radioisotope to an antibody with minimum chemical or immunological damage to the antibody is a necessity. Until recently, $^{125}\text{I}$ was the isotope of choice for labeling antibodies to be used for diagnostic and therapeutic purposes. As radioactive antibodies, they are able to block vascular permeability, inhibit cell proliferation, and mediate tumor cell killing by approximately two orders of magnitude. A 5-fold increase in the molar ratio of $^{125}\text{I}$-labeled 5E8 reduced the amount of antibody required to inhibit 50% of the tumor colony formation from 0.1 to 0.001 $\mu$g/10^3 cells. It is of interest to note that no increase in the efficiency of tumor killing was apparent with $^{125}\text{I}$-labeled 5E8 when the molar ratio was greater than 1.7.

Unlabeled 5E8 Inhibition of Tumor Killing by $^{125}\text{I}$-labeled 5E8. In an attempt to demonstrate the specificity of tumor killing by $^{125}\text{I}$-labeled 5E8 and to sustain the notion that the cytotoxicity is due to the radioactivity of the antitumor antibody, A549 cells were incubated with increasing amounts of unlabeled 5E8 together with $5 \times 10^5$ A549 cells were incubated for 5 min at 4°C with these mixtures. After removal of the unbound $^{125}\text{I}$-labeled 5E8, the pulsed cells were placed at $1 \times 10^5$ cells/well in the top layer of the agar, andish a colony forming assay was performed. Colonies were counted and the percentage of inhibition of colony formation was determined. Points, average of triplicate experiments.

Effect of Second Antibody upon $^{125}\text{I}$-labeled 5E8 Mediated Suppression of Tumor Colony Formation. While it is recognized that radiolabeled antibodies are able to kill cells without being internalized, it is possible that internalization of the $^{125}\text{I}$-labeled 5E8 by A549 cells would increase the efficiency of tumor cell killing due to the increased proximity of the radioactivity to the DNA of the cells. We have previously established that HRP-labeled 5E8 is internalized following its binding to A549 cells and that the amount of HRP-labeled 5E8 internalized is increased (up to 25%) by the addition of a second antibody (rabbit anti-mouse immunoglobulin) that is capable of cross-linking the HRP-labeled 5E8 on the surface of the tumor cells (19). In view of these results, an attempt was made to determine what effect this second antibody would have on tumor killing when added to A549 cells after a pulse with $^{125}\text{I}$-labeled 5E8. Accordingly, A549 cells were pulsed with various amounts of $^{125}\text{I}$-labeled 5E8 and the cells were subsequently cultured in the presence or absence of a second antibody (affinity column purified rabbit anti-mouse immunoglobulin). No significant difference was observed in the inhibition of A549 colony formation when the $^{125}\text{I}$-labeled 5E8 pulsed tumor cells were cultivated with or without the second antibody (Fig. 7). While these results indicate that the expected increase in $^{125}\text{I}$-labeled 5E8 internalization did not enhance tumor killing, these results do not gainsay the possibility that the internalization of 5E8 which occurs in the absence of the second antibody does contribute to the ability of $^{125}\text{I}$-labeled 5E8 antibody to suppress the proliferation of tumor cells.

DISCUSSION

The ability to successfully conjugate a radioisotope to an antibody with minimum chemical or immunological damage to the antibody is a necessity. Until recently, $^{125}\text{I}$ was the isotope of choice for labeling antibodies to be used for diagnostic and
therapeutic applications (20–25). However, the relatively short half-life of $^{131}$I-labeled antibody and its poor imaging, unfavorable γ-ray emissions, and myelosuppressive activity, have limited the clinical use of this isotope (26). A variety of methods to attach metallic isotopes to the antibody via chelator bridge have been developed in the last 10 years making these molecules viable alternatives to the use of iodine. These radiolabeled antibodies have been investigated extensively to determine which of the radionuclides are optimal for use in radioimaging and radioimmunotherapy (27–33). However, the metallic radioisotopes are not always superior to radioiodine for radioimmunoimaging (34), and radioiodine remains the most commonly utilized radioisotope for imaging and targeting (26). Bloomer et al. (23) reported that $^{125}$I would not have any great applicability for radioimmunotherapy because Auger emitters such as $^{125}$I were cytotoxic only when localized within close proximity to the genome. On the contrary, the successful outcome of immunolymphoscintigraphy with $^{131}$I-labeled antibody in animal experiments was reported (35, 36), and Boven et al. (25) demonstrated substantial radiation damage by $^{125}$I-labeled monoclonal antibody with in vitro experiments suggesting that $^{125}$I-labeled antibody remains one of the potential viable agents for clinical radioimmunotherapy.

In this study, the in vitro growth inhibitory potential of $^{125}$I-labeled 6E8 on human lung carcinoma cell lines was investigated in detail. It has been established here that radiolabeled anti-gpl60 antibody kills gpl60 positive tumor cells selectively and dose dependently when the tumor cells are pulsed with the antibody prior to cultivation in soft agar. The failure of the anti-gpl60 antibodies to kill gpl60 negative tumors was shown not to be due to their relative radioreistance since the colony formation by the control tumor cells and gp160 positive tumors were both suppressed when the $^{125}$I-labeled 6E8 was present chronically, i.e., throughout the culture period of the colony forming assay.

After establishing that the unlabeled 6E8 antibody has no effect upon the growth of the tumor targets and that the unlabeled 6E8 antibody effectively blocks the selective killing of the tumor cells by $^{125}$I-labeled 6E8, we have concluded that the radiolabeled antibody is required to bind to the cell surface gp160 molecule and that the cytotoxicity is mediated by the radioactivity associated with the labeled antibody. Since we have previously demonstrated that approximately 40% of the 6E8 bound to A549 cells is internalized 30 min after binding (19), it is possible that internalization of $^{125}$I-labeled 6E8 contributes to its ability to kill the tumor targets. While other radioisotopes such as $^{186}$Re and $^{90}$Y which have intermediate-energy β emission probably do not require internalization, the lower energy associated with $^{125}$I suggests that internalization may very well effect the ability of $^{125}$I-labeled antibody to kill tumor cells. Other investigators have argued that $^{125}$I must be in close proximity to the nucleus in order to kill the cell (7). Boven et al. (25) indicated that they anticipated that an increased internalization of $^{125}$I would bring about greater cytotoxicity even though they observed killing of tumor cells with $^{125}$I-labeled antibodies which bound to the surface of the tumors with little or no subsequent internalization (25). While we were unable to increase tumor killing efficiency with the addition of a second antibody, it is still likely that the internalization which occurs without the addition of the second antibody was sufficient for tumor killing to occur. The recognition by others that $^{125}$I is liberated following internalization (37) further confuses the issue of the role internalization plays in radioimmunotherapy with $^{125}$I.

In addition to establishing that $^{125}$I-labeled 6E8 kills tumor targets selectively, we have determined that the efficiency of this killing can be enhanced by increasing the molar ratio of the $^{125}$I to 6E8. Furthermore, we determined that two molecules of the radiolabeled iodine could be coupled to one monoclonal antibody without noticeably altering the antibody's ability to bind to the surface of the tumor. When the molar ratio exceeded two (i.e., 2.5 or greater) the antibody binding activity was impaired. This may be a rather general if not a universal finding since a previous report using polyclonal antibodies also noted that little or no change occurred in the antibody's binding activity provided the number of atoms of $^{125}$I per molecule of antibody did not exceed two (38).

Two related findings made with the $^{125}$I-labeled 6E8 killing of gp160 positive tumor cells will require further consideration: (a) it was noted that in all three of the tumors only partial killing was observed; and (b) it was apparent that more of the A549 cells were killed than either Calu-3 or A427. The latter could be due, among other things, to differences in the cell's radiosensitivity, to the efficiency of internalization of antibody-gp160 complexes, or to the proportion of cells expressing gp160 on their surface. The first two possibilities have been ruled out and the latter possibility (differences in the proportion of cells expressing gp160) is currently under further investigation. As indicated in Table 1, a greater percentage (85%) of A549 cells expressed gp160 than did either Calu-3 (69%) or A427 (50%). However, if failure to express the gp160 were the only reason for the cells within each tumor type to escape the radioimmunotherapy then one would expect that the colonies derived from the tumor cells treated with $^{125}$I-labeled 6E8 to consist largely if not exclusively of gp160 negative tumor variants. Preliminary experiments only partially support this notion. While the proportion of gp160 negative cells in the colonies obtained from antibody treated cultures are considerably greater than control cultures, the lack of gp160 in these cells appears to be a transient phenomenon. Thus, cells that are escaping the radioimmunotherapy may be able to either modulate the gp160 off of their surface or may transiently lack gp160 due to differential expression perhaps occurring at various times in the cell cycle. These and other possibilities must be investigated.

We recognize that the radiolabeled antibody's ability to kill tumor cells selectively in vitro may not, for a variety of reasons, be recapitulated in vivo. However, the knowledge gained from our in vitro radioimmunotherapy studies have helped us considerably in designing our in vivo studies which are currently in progress. In these studies, we are using severe combined immunodeficient mice which have been shown to support the growth of the gp160 positive lung tumors both s.c. and in the lung (39). While the in vivo studies are still very preliminary, we have observed marked changes in tumor growth in vivo following a single injection of $^{125}$I-labeled 6E8 into tumor bearing severe combined immunodeficient mice.

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