In vitro Toxicity of A-431 Carcinoma Cells with Antibodies to Epidermal Growth Factor Receptor and Epithelial Glycoprotein-1 Conjugated to Radionuclides Emitting Low-Energy Electrons

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ABSTRACT

Purpose: The ability of antibodies (Abs) conjugated to radionuclides emitting low-energy electrons to specifically kill nonadherent lymphoma target cells in vitro was demonstrated previously. This study extends this work to adherent carcinoma cells. The fact that these cells are spread out on plastic can potentially make it more difficult to deliver radiation to the nucleus from decays in the cytoplasm or on the cell surface.

Experimental Design: The Abs tested were anti-epidermal growth factor receptor and anti-epithelial glycoprotein-1, conjugated to indium-111 or iodine-125, which emit low-energy Auger and conversion electrons. Conjugates of the β-particle emitter, iodine-131, also were tested, for comparison. Abs were incubated with the cells for 2 days, and then the treated cells were assayed for colony-forming units. The radiation dose delivered to the nucleus was calculated from the cumulative decays per cell.

Results: With conjugates of 111In, very potent killing was obtained with both of the Abs, with 100% kill (approximately 4–5 logs) even at subsaturating Ab concentrations. Lower levels of kill were obtained with 125I or 131I conjugates. Conjugates with 131I, a β-particle emitter, produced greater nonspecific toxicity. The greater potency of 111In could be attributed to the higher specific activity that was obtained routinely with this radiolabel, up to 70 mCi/mg. Uptake of radioactivity peaked at approximately 200 cpm per cell. Dosimetry calculations, using subcellular S values, demonstrated that the toxicity observed was consistent with the amount of radiation delivered to the nucleus.

Conclusions: These results are similar to previous results obtained with B lymphoma cells and indicate that this approach is applicable to a wide range of tumor types. Radionuclides emitting low-energy electrons are effective at killing target cells with relatively little nonspecific toxicity, if sufficient activity is delivered to the cell. Most Abs to high-density cell surface antigens would probably be effective.

INTRODUCTION

We have been investigating the potential role of radionuclides emitting low-energy electrons (LEEs), conjugated to antibodies (Abs), for radioimmunotherapy. The advantage of such radionuclides, compared with β-particle emitters, which are most widely used for this purpose, is the much shorter path-length in tissue of the electrons emitted. A consequence of this shorter path-length is that the targeted cells can be killed with less toxicity to normal tissue. This was demonstrated in a severe combined immunodeficient mouse xenograft model system for disseminated human B-cell lymphoma: mice were cured with indium-111 or gallium-67 conjugates under conditions in which yttrium-90 conjugates were ineffective (1). The results could be attributed to the fact that the maximum tolerated dose was approximately 10-fold higher for the LEE emitters (111In and 67Ga) than for the β-particle emitter (90Y). β-Particles seem appropriate to kill substantial tumor masses, especially if the Ab is distributed heterogeneously within the tumor, but it is well established that as the tumor size becomes smaller, β-particles become increasingly less effective as toxic agents because only a small fraction of the radiation is absorbed by the target cells (2–4). LEEs seem more appropriate to target micrometastases. The optimal energy for single cell kill for radionuclides deposited on the cell surface is approximately 20–30 keV (2).

The term “LEEs” is used to refer to Auger electrons and some of the internal conversion electrons that are emitted by radionuclides decaying by electron capture or internal conversion. Some conversion electrons have relatively high energies (>100 keV), and these will not have the localized energy deposition that is desired. Because most LEE emitters produce a large number of electrons (as many as 20 or more) of varying energy and abundance, the selection of the optimal radionuclide is complex. Most of the abundant Auger electrons have energies < 5 keV, and these are probably irrelevant because they essentially cannot reach the nucleus from the cytoplasm. The best approach to compare LEE emitters is with the subcellular S values, published in 1997 in MIRD Cellular S Values (5). However, it should be noted that many radionuclides emitting abundant LEEs are not included in this book. In practice, we are limited to radionuclides that are commercially available, namely 111In, 67Ga, and iodine-125, all of which are relatively poor LEE...
emitters. A number of radionuclides have been identified that would be many times more potent than these (6). The disadvantage of LEEs is that a large number of decays is required to kill a cell. However, this is not necessarily a problem for Abs to high-density antigens, which include many of the Abs that are most widely used in experimental cancer therapy.

One practical advantage of using LEE emitters is that target cells can be killed efficiently in vitro with the Ab conjugates. In contrast, Ab conjugates with β-particle emitters, although used extensively in animal therapy experiments and in patients, are generally not even tested for in vitro toxicity, partly because earlier studies demonstrated that they typically display very little, if any, killing (discussed in ref. 7) and also because their toxicity is known to be due primarily to cross-fire, meaning radiation emitted from nearby cells, as opposed to radiation emitted from the target cell itself. With B-cell lymphomas, we demonstrated efficient in vitro killing with Abs conjugated to 111In, 67Ga, 125I, and technetium-99m (7–9). The in vitro toxicity assays were also performed with β-particle emitters, which demonstrated that the key difference between LEEs and high-energy electrons was not in their ability to kill individual target cells (which could also be achieved with the β-particles) but in the level of nonspecific toxicity, which was much greater with the β-particles than with the LEEs (8). This difference in nonspecific toxicity between β-particles and LEEs was also demonstrated in vivo, as noted above.

As a continuation of this work, we have extended the experiments to other tumor types, namely, the carcinoma cell line A-431. This is an adherent cell line, unlike the nonadherent B-cell lymphomas, which is an important distinction in this context. Because adherent cells are spread out on the plastic substrate, the average distance from cell surface to nucleus or from cytoplasm to nucleus is considerably greater than that with a nonadherent cell. Because the cell is killed by radiation energy deposited in the nucleus, this factor will make it more difficult to kill adherent cells with LEEs. The impact of this factor is difficult to predict and depends on the size, shape, and relative location of the cell membrane and the nucleus, but it could be substantial. Accordingly, in this study we have attempted to kill A-431 cells with Abs conjugated to 111In, 125I, and 131I (a β-particle emitter, for comparison). The Abs used were selected for their reactivity with high-density antigens on this cell line and for their clinical relevance. Accordingly, we have used Abs to epidermal growth factor (EGFr), a widely used tumor marker (10, 11). It was important to also test Abs reacting with other high-density antigens to determine whether the results are generally applicable. Accordingly, anti-epithelial glycoprotein-1 (EGP-1) was also tested.

There were two major previous attempts to use Abs conjugated to LEE emitters as therapeutic agents (12–15); in one of these cases, an anti-EGFr Ab was used for therapy of gliomas (13, 14). Experimental results were promising, but therapeutic effects in patients were not impressive. However, those studies used 125I, which has a half-life (60 days) that is probably too long to be optimal for cell toxicity. In addition, it is now recognized that a conventional 125I label leaves the cell rapidly after the Ab carrier is internalized, due to catabolism of the Ab, and that this does not occur with most radiometals, such as 111In (which are therefore called residualizing radiolabels; ref. 16).

### MATERIALS AND METHODS

#### Cell Lines, Antibodies, and Radiolabeling

The A-431 cell line is a vulvar squamous carcinoma and was obtained from the American Type Culture Collection (Manassas, VA). It was grown as described previously (17). Hybridomas 225 and 528, producing Abs reactive with EGFr (11), were obtained from American Type Culture Collection. They compete for the binding site of epidermal growth factor and prevent cell stimulation by epidermal growth factor binding. The Ab subclass is IgG1 and IgG2a, respectively. Hybridoma RS7, producing an IgG1 Ab to EGP-1, was described previously (18). The nonreactive control Ab used in most experiments was MX352a, an IgG2a; however, an IgG1 nonreactive Ab, MOPC-21, was also tested in some experiments: The uptake of both of these control Abs by A-431 cells was essentially the same and extremely low. Hybridomas were grown in tissue culture, and Ab was purified from 3 to 4 liters of spent medium by chromatography on protein A-Sepharose (Amersham Pharmacia, Piscataway, NJ) by standard methods. Abs were labeled with 125I and 131I by the chloramine-T method and labeled with 111In with the chelator isothiocyanato-benzyl-diethylenetriaminepentaacetic acid, as described previously in detail (7). Labeled preparations were analyzed by instant thin-layer chromatography, gel filtration high-performance liquid chromatography, or both, by methods that have been described previously (19), to determine the level of radioactivity not bound to the Ab, which was always <10% and usually <5%. Representative preparations of radiolabeled Abs with each radiolabel were tested for immunoreactivity (percentage bindable) by incubation with a large excess of cells. Control tubes had unlabeled unlabeled Ab added to block specific binding and therefore indicate the level of nonspecific binding: Specific binding was calculated by subtraction. The peak specific binding was as follows: 125I-225, 61.4%; 111In-225, 41.6%; 125I-RS7, 60.7%; and 111In-RS7, 54.1%.

#### Antibody Processing Experiments

These methods were described previously in detail (17). Briefly, cells were plated 1 day before use in a 96-well plate. Radiolabeled Ab was then added and incubated for 2 hours at 37°C. Control wells in every experiment contained a large excess of unlabeled Ab to demonstrate the specificity of Ab binding, which was at least 90% specific. After washing the wells, tissue culture medium was added, and incubation was continued for 2 to 3 days. At various time points, as indicated, 100 μL of supernatant were collected, counted for radioactivity, and then precipitated with cold 10% trichloroacetic acid (TCA) to distinguish between intact (TCA precipitable) and degraded (TCA soluble) cpm in the supernatant. The cells were collected after further washing by solubilizing with 2.0 mol/L NaOH and harvesting on a cotton swab, and then they were assayed for cell-bound cpm.

#### Antibody Uptake at Saturation in a Prolonged Antibody Incubation

These assays also were described previously in detail (20). Briefly, cells were incubated continuously with near-saturating concentrations of radiolabeled Ab. The Ab concentrations required were selected in preliminary experiments. At various time points, for 2 to 3 days, cell-bound cpm was determined. From the specific activity of the Ab used, and correcting for the percentage of non–protein-bound radioactivity (always <10%), the number of Ab molecules bound was cal-
culated. From cell counts, determined from other wells, the number of Ab molecules bound per cell was determined.

Toxicity Assays. Cells (2 \times 10^4) were plated in 96-well plates in 0.15 mL and allowed to adhere for 1 day before application of the Abs. After aspirating the medium, serial dilutions of Ab were added, also in 0.15 mL, and plates were incubated for 2 days at 37°C. Control wells had no Ab added. At day 2, 1 well of each set was used for cloning. The cells were suspended by trypsinization, pelleted, and resuspended in 5.33 mL. Five serial 4-fold dilutions were prepared, by transferring 1.33 mL into 4.0 mL. All 4.0 mL of each dilution were plated in a 60-mm-diameter Petri dish and incubated at 37°C. A cell count was obtained from another control well to calculate the cloning efficiency. Bound radioactivity was determined from additional wells by harvesting on a cotton swab, as described above. In some cases, with 111In-labeled Ab, the counts per well were >10^6; because such high cpm are not counted accurately, these samples were allowed to decay, usually for 1 week, and then recounted. The cpm at time 0 was then calculated from the decay curve.

At day 1 to 3 after plating, depending on the day of the week that the assay was started, the cells were overlaid with 4.0 mL of 0.5% agar to prevent any cells from floating away from the primary colonies. The agar solution contained Medium 199, 20% fetal bovine serum, penicillin/streptomycin, glutamine, and sodium pyruvate (all from Life Technologies, Inc., Rockville, MD) and was maintained at 42°C until use. After the agar hardened (approximately 30 minutes), 4 mL of standard medium were added. Plates were incubated for 17 to 18 days. The agar was removed by rimming with a spatula, and colonies were fixed with formaldehyde and stained with methylene blue as described previously (21). The cells grow normally under the agar overlay, with no indication that the agar inhibits either the spreading of colonies or the growth rate of the cells, and the cells had entirely normal morphology.

In some experiments, cell-bound cpm and viable cell counts were determined at various time points. Cpm were obtained in triplicate by solubilizing with 2.0 mol/L NaOH, as described above. Cell counts were from wells trypsinized as described above. To obtain data from time points later than day 2, it was necessary to transfer the cells to larger wells because they had become near confluent in the original 96-well plate. The aim was to maintain the cells under conditions of rapid growth, similar to the conditions present in the Petri dishes used for cloning. Therefore, individual wells were trypsinized as described above, pelleted, resuspended, and transferred to a 24-well plate. These secondary wells were then harvested for both cpm and viable cell counts at the desired time points (usually days 3 and 6).

Uptake of Radioactivity and Dosimetry Calculations. To calculate radiation dose delivered, it is necessary to know the cumulative decays per cell and the S value. The cpm per cell, at various time points, was determined as described above, and the cumulative cpm was calculated as the area under the curve. The number of disintegrations was calculated from the gamma counter efficiency for 111In, which was 70.9%. The subcellular S values were from MIRD Cellular S Values (5). There are a number of uncertainties involved in the choice of an S value, as discussed under Results. For comparison with the radiolabeled Abs, cells were irradiated with a cesium-137 irradiator (J. L. Sheppard & Associates, San Fernando, CA) at the Radiation Safety Department, University of Medicine and Dentistry of New Jersey (Newark, NJ). The cells were irradiated while adherent to flasks. After irradiation, the medium was changed, and the cells were incubated overnight before assaying colony-forming units (CFU) under the same conditions used in the other experiments.

Radiation doses were also calculated for nonspecific toxicity from 131I; that is, from decays occurring in the medium. The highest concentration tested was 269 μCi/mL (this high concentration was used with a nonreactive control Ab to demonstrate nonspecific toxicity; much lower doses were used with the reactive Abs). Assuming 100% absorption of the electron energy, the radiation dose would be 2,405 cGy, using the method described previously (8). However, not all of the energy will be absorbed by the small incubation volume of 0.15 mL; the estimated fraction absorbed is 89.6% (22). Also, cells at the edges of the wells will receive a lower dose than cells in the center, by a factor of approximately 2. Therefore, the estimated dose is 1,080–2,160 cGy, depending on the location of the cell in the well.

RESULTS

Selection of Antibodies Binding at High Levels to A-431 Cells and Antibody Processing Experiments. In preliminary experiments, we identified four Abs reacting with high-density antigens: anti-EGFr, anti-HLA class I (W6/32), anti-EGP-1, and anti-CD147. Fig. 1 shows the results of Ab processing experiments with these Abs over a period of 3 days. All of the Abs tested, like nearly all of the Abs tested previously on other cell lines (17, 23, 24), had a low level of dissociation that was essentially complete in 4 hours. The peak dissociation was ±14% for three of the Abs and 25% for W6/32. The remaining bound Ab was gradually catabolized at a rate that depended on the particular Ab. The catabolism of anti-EGFr was significantly faster than that of anti-CD147 but slower than that of the other two Abs. Cell retention of anti-EGFr seems high enough such that nonresidualizing radiolabels (iodine labels) may have toxic activity, but a residualizing label would be expected to provide some advantage. We note that such rapid catabolism of W6/32 is not generally seen (17, 23, 25), so evidently this represents an unusual characteristic of this cell line. The results shown were obtained with the anti-EGFr Ab 528, but essentially the same results were obtained with the other EGFr Ab, 225.

Antibody Uptake in a Prolonged Antibody Incubation with a Saturating Concentration of Antibody. We next determined the level of Ab uptake per cell in a prolonged Ab incubation, out to 2 to 3 days, using a saturating concentration of anti-EGFr or anti-EGP-1 Abs. The conditions were identical to those that would later be used in toxicity experiments, except that only a trace radiolabel was used. We note that this difference is likely to have some impact because cells are damaged and killed, the level of Ab uptake and retention is likely to be affected. Fig. 2 shows representative results. In each of these experiments, a 2-fold higher Ab concentration was also tested, and there was, at most, a minor increase in the Ab molecules bound, which demonstrates that near-saturation was reached.
Abs were labeled with either $^{125}$I or $^{111}$In. Because some of the bound cpm will be in the form of catabolites, especially with $^{111}$In, we refer to the bound Ab as “Ab equivalents.” With anti-EGFr (Fig. 2A), $^{125}$I uptake peaked early, at 4 hours, with approximately $1.8 \times 10^6$ Ab molecules per cell; at later time points, the bound Ab equivalents decreased gradually yet still remained at a relatively high level for at least 2 days. When an $^{111}$In label was used, this gradual decrease did not occur; instead, the bound Ab equivalents increased slightly with time. This seems consistent with the Ab processing experiments described above, which showed a low level of catabolism of anti-EGFr. The peak uptake of $^{111}$In, at 44 hours, was $2.57 \times 10^6$ Ab equivalents per cell.

Similar experiments with anti-EGP-1 are shown in Fig. 2B. With this Ab conjugated to $^{111}$In, the peak uptake was even higher than that with anti-EGFr, with $6.96 \times 10^6$ Ab equivalents bound per cell. Again, uptake of $^{111}$In was substantially higher than uptake of $^{125}$I, presumably due to catabolism of the Ab. One difference with anti-EGP-1, in comparison with anti-EGFr, is that with the $^{125}$I label, binding was still relatively low at 4 hours and required 23 hours to reach a near-plateau. A possible explanation for this result is that the Ab accumulates inside the cell in a noncatabolic compartment. Preliminary immunohistochemistry experiments support this possibility because we found anti-EGP-1 localization to perinuclear cytoplasmic vesicles.  

Similar experiments with the two other Abs reacting with high-density antigens on A-431 cells were also performed. With $^{125}$I-anti-CD147, the peak uptake was $8.9 \times 10^6$ Ab equivalents per cell, which was reached at 23 hours. With $^{125}$I-W6/32, the peak uptake was $3.1 \times 10^6$ Ab equivalents per cell, which was reached at 47 hours. Given the relatively rapid catabolism of W6/32, as shown above, uptake of an $^{111}$In label would be expected to be considerably higher, but this has not yet been tested. For subsequent toxicity experiments, we focused on anti-EGP-1 and anti-EGFr because diethylenetriaminepentaacetate acid conjugates of these two Abs were available for labeling with $^{111}$In.

**Cytotoxicity of A-431 Cells with Radiolabeled Antibodies.** Toxicity experiments were performed with $^{111}$In, $^{125}$I, and $^{131}$I conjugates using serial dilutions of Abs. The β-particle emitter, $^{131}$I, was included for comparison with the two LEE emitters. Based on the uptake experiments described above, the nonresidualizing iodine labels would be expected to be less efficient than a residualizing label such as $^{111}$In, but the uptake of the iodine labels was high enough such that killing might occur. One advantage of the conventional iodine labels is that they are the easiest and least expensive labels to prepare. In control experiments, the unconjugated Abs were also tested. Whereas anti-EGFr has a strong therapeutic effect in vivo, it only weakly inhibits cell growth in vitro (26). Using unconjugated Abs at 5 µg/mL, which is as high as any concentration that was tested in toxicity assays with radiolabeled Abs, the fraction surviving was 0.61 for anti-EGFr and 0.69 for anti-EGP-1, which is consistent with previously published data (26). We conclude that the Abs used, unconjugated, have very little or no toxic effect.

Fig. 3A shows cytotoxicity data obtained with $^{125}$I-labeled Ab conjugates, including a nonreactive control Ab. Moderately effective killing with both Abs was obtained, which was specific. With anti-EGFr, the maximum kill was approximately 93% (at 40 µCi/mL), and with anti-EGP-1, the maximum kill was approximately 97% (at 98 µCi/mL). With both Abs, killing leveled off at higher concentrations, which can be attributed to the fact that antigen sites were saturated. This was demonstrated by assays showing the bound cpm in these experiments: As the Ab concentration increased to the highest concentrations tested, there was very little increase in the cpm bound (data are shown below). An implication of these results is that stronger killing would be achieved if a higher specific activity could be attained. By comparing the nonreactive Ab with the two reactive Abs, we can estimate how much more effective the reactive Ab was: It was $154 \times$ more active for anti-EGFr and $36 \times$ more active for anti-EGP-1.

Killing was much more potent with the $^{111}$In label, as

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1 M. Mattes, unpublished data.
shown in Fig. 3B. With both Abs tested, 100% killing was obtained at the highest Ab concentrations, and this killing was highly specific, as shown by comparison with a labeled nonreactive Ab. The advantage of 111In over 125I can be attributed to two factors. First, the specific activity was greater for 111In, by approximately 3- to 5-fold, being in the range of 53–66 mCi/mg. Secondly, 111In is a residualizing radiolabel, as noted above, and this is significant because there is substantial catabolism of both of these Abs. The use of a residualizing label not only causes increased accumulation of cpm/cell but also causes much of the retained radioactivity to be present in the cytoplasm (within lysosomes) and hence closer to the nucleus, which may increase the effectiveness of LEEs. Although our data with lymphoma cells indicate that the subcellular localization of the radioactivity is not an important factor (9), it still can be expected to provide at least a small advantage. The advantage of the reactive Ab versus the nonreactive Ab for the 111In labels was 57X for anti-EGFr and 84X for anti-EGP-1. Whereas this ratio is lower than that for 125I with anti-EGFr, it should be emphasized that 111In-anti-EGFr killed 100% of the cells, whereas 125I-anti-EGFr killed only 93% at the maximum saturating concentration.

Results with 131I are shown in Fig. 3C. As expected from our previous studies, 131I-labeled Ab conjugates also killed cells with some specificity but had much greater nonspecific toxicity than the LEE emitters. The nonspecific toxicity is due to decays occurring in the medium because the β-particles will reach the cell nucleus from the medium. Thus, at a concentration of 269 μCi/mL, 131I-labeled control Ab killed cells very effectively, with a fraction surviving of 6.06 × 10⁻⁴. In contrast, control Ab conjugates with LEE emitters produced much less toxicity at high concentrations. With 125I, 443 μCi/mL produced a fraction surviving of 0.356; with 111In, 321 μCi/mL produced a fraction surviving of 0.358. The estimated radiation dose from nonreactive Ab.
tive $^{131}$I-labeled Ab was calculated as described in Materials and Methods. At the highest Ab concentration tested (269 $\mu$Ci/mL), the dose was calculated to be 1,080–2,160 cGy (depending on whether a cell was at the center or the edge of the well). This dose would produce a fraction surviving of $1.99 \times 10^{-3}$ to $3.02 \times 10^{-6}$ [based on the $D_0$ (the dose required for 63% kill in the linear part of the dose-response curve) and the extrapolation number from $^{131}$Cs irradiation experiments, which are described below]. Thus, these calculations are consistent with the toxicity results observed. In a comparison of the reactive and nonreactive Abs, anti-EGP-1 was only 2.6× more potent than the nonreactive Ab, whereas anti-EGFr was 17.4× more potent. It appears from Fig. 3 that anti-EGP-1 is relatively less effective with the $\beta$-particle emitter $^{131}$I than with LEE emitters; a possible reason for this is discussed below.

Fig. 4 shows the level of cpm uptake in the same experiments shown in Fig. 3. Uptake was determined only at day 2, at the time of cloning; thus, it may not reflect total cumulative decays over the course of the experiment. Several points should be emphasized. First, a plateau in the binding of $^{125}$I and $^{131}$I is shown, indicating that antigen sites were saturated, and this is why increased Ab concentrations did not result in increased killing. Second, uptake was fairly similar for both of the Abs tested, with all three radionuclides. Third, uptake of $^{111}$In was very high, with $2.3–3.6 \times 10^5$ cpm bound per well of a 96-well plate. This is due to the much higher specific activities of the $^{111}$In conjugates. Fourth, although uptake of $^{131}$I was much lower than uptake of $^{111}$In, the $^{131}$I-anti-EGFr still killed cells very effectively, as shown in Fig. 3. This indicates that fewer decays of cell-bound $^{131}$I are required for effective cell kill, in comparison with $^{111}$In. Similar results were obtained previously with lymphoma target cells. This difference is at least partially due to the nonspecific irradiation of cells from $^{131}$I in the medium, which was discussed above. Fifth, although uptake of $^{131}$I-anti-EGFr and $^{131}$I-anti-EGP-1 was very similar, the former killed much more effectively. This result can tentatively be explained by the different rates of accumulation of cpm, as shown in Fig. 2. Those experiments used $^{125}$I, but accumulation of $^{131}$I is expected to be similar. As shown, accumulation of iodinated anti-EGP-1 was gradual, with accumulation at day 2 being much higher than the accumulation at 4 hours. Therefore, the uptake at day 2 will be much higher than the average uptake over the 2-day period. In contrast, accumulation of iodinated anti-EGFr peaked at 4 hours and was considerably lower at day 2. Thus, the uptake at day 2 is expected to underestimate the average uptake over the 2-day period.

**Dosimetry.** To calculate the radiation dose delivered to the cells, it is necessary to know the cpm at various time points, as well as the number of cells at each time point. Therefore, additional experiments were performed in which bound cpm and the cell number were determined at various times. This study was done with only an $^{111}$In label because this was adequate to answer the questions of interest. For comparison, the sensitivity of A-431 cells to external beam irradiation was also determined. For this study, Ab concentrations were tested that covered a range in fraction surviving from 0.01 to 0.001, based on the previous experiments. If killing is too strong in these experiments, there are too few viable cells remaining at the later time points to obtain accurate cell counts. Fig. 5 shows typical cell growth curves in these experiments: It is important to note that by day 6, there was significant killing of cells at the higher Ab concentrations. Because the target cells are adherent and the wells are washed before harvesting, we assume that dead cells detach and are washed away; this is generally consistent with the results obtained, but there were some exceptions, in which the viable cell counts decreased more rapidly than the bound cpm, suggesting that certain types of dead cells may remain attached to the plate for some time interval. Fig. 6 shows the cpm bound per cell at various time points for both Abs. The peak uptake was as high as 158 cpm/cell, and it should be again pointed out that the Ab concentrations used were selected such that the fraction surviving was at least $~0.001$. Thus, uptake of cpm would be even higher under conditions of 100% kill.

The radiation dose delivered per cell was calculated from the cumulative decays per cell and the $S$ value. The $S$ value is a function of the location of the decay within the cell, and there

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Fig. 4  Cpm uptake in the same experiments shown in Fig. 3. Bound cpm were collected after 2 days of incubation with the Ab by solubilization with 2.0 mol/L NaOH and are expressed as cpm/well. The number of cells plated per well, 1 day before addition of Ab, was $2 \times 10^3$. Results are shown for anti-EGFr (□), anti-EGP-1 (△), and a control nonreactive Ab (○), labeled with $^{125}$I (A), $^{111}$In (B), or $^{131}$I (C). Results shown are representative of two experiments performed with each radiolabeled Ab.
is some uncertainty regarding the best value to use. The available choices are the cell surface or the cytoplasm. Although we could estimate what fraction of decays are from the cell surface, based on the processing experiments described above, our previous work with lymphomas indicated that it is probably more useful to use the cytoplasmic S value (9). This is because the calculated S values are based on a model of the cell in which the nucleus and the cell membrane are concentric spheres, whereas, in reality, the nucleus is usually on one side of the cell (this was confirmed with A-431 cells by staining the nucleus with Hoechst 33342). This difference means that much of the nucleus is closer to the cell surface than would be expected from the concentric sphere model and decreases the actual difference between the cell surface and the cytoplasmic S values. The S value also depends on the size and shape of the cell and the nucleus. In suspension, after trypsinization, the mean diameter of A-431 cells was determined to be 14.1 μm, and the mean nuclear diameter was determined to be 10.6 μm. However, when adherent to plastic, as in the cytotoxicity assays, A-431 cells spread out and form clusters with a cobblestone-like pattern. The average diameter of A-431 cells 1 day after plating (as in the toxicity assay) was 27.2 μm. But this does not consider the vertical height of the cell, which was not measured but is certainly much less than the length or width. Because, to our knowledge, there are no published S values for cells of this shape, our only alternative is to use the S values for round cells. This is probably an overestimate of the true S value, but the difference may not be large. The published S values are 1.65 × 10⁻² cGy/Bq second for decays in the cytoplasm and 1.07 × 10⁻² cGy/Bq second for decays on the cell surface.
cumulative decays and the calculated rad dose. Cumulative decays were calculated over only 3 days, although data were also obtained at day 6, because the cell counts were too low to be accurate at day 6, at the higher Ab concentrations (which produced an ultimate fraction surviving close to 0.001). Therefore, the total dose will be somewhat higher than the 3-day dose, but this difference will be relatively small and can be taken into account: At the lower Ab concentrations, for which reliable cell counts could be obtained at day 6, the cumulative disintegrations at day 6 were, on average, 45% higher than the cumulative disintegrations at day 3 (the range was 20% to 63%). The mean $D_0$ was 1,346 cGy (81,599 decays) for anti-EGFr and 1,062 cGy (64,321 decays) for anti-EGP-1. For comparison, the sensitivity of A-431 cells to external beam irradiation (with $^{137}$Cs) is also shown (Fig. 7A and B). For $^{137}$Cs irradiation, the $D_0$ was 166 cGy, and the extrapolation number was 3.03. Thus, these cells appear to be typical of most epithelial cell lines in their radiation sensitivity (27). The toxicity per rad of $^{111}$In-labeled Ab was lower than the toxicity per rad of external beam radiation by approximately 6- to 8-fold, which can be explained by factors that are discussed below.

DISCUSSION

This study has demonstrated that Abs linked to LEE emitters can efficiently kill adherent A-431 cells in vitro; 100% kill (of approximately $4 \times 10^4$ cells) was obtained at sufficiently high Ab concentrations. Because similar results were previously obtained with lymphoma cells, this approach appears not to be restricted by the size and shape of the target cell, although the flattened shape of adherent cells is likely to make this type of killing somewhat more difficult. The potency can be attributed to the high level of Ab uptake, reaching levels as high as 200 cpm per cell, which is somewhat higher than that obtained with lymphoma target cells. Killing was obtained with both of the Abs tested, anti-EGFr and anti-EGP-1. Considering the similarity of the dose-response curve for both of these Abs, it is likely that similar results would be obtained with other Abs that bind at comparable levels to this cell line. Specific killing was obtained with both of the LEE emitters tested, $^{125}$I and $^{111}$In, but was much more potent with $^{111}$In. This, again, is similar to the results obtained with lymphoma cells, and it can be attributed to the higher specific activity of the $^{111}$In conjugates. Specific killing was also obtained with the $\beta$-particle emitter $^{131}$I: This radionuclide was very efficient at cell kill but had the disadvantage of greater nonspecific toxicity, which can be attributed to decays occurring in the medium and the long path-length of the $\beta$-particles. Because the sensitivity of A-431 cells to external beam irradiation was typical of most tumor cells, it is likely that similar results would be obtained with other cell lines. The dosimetry calculations suggest that the amount of $^{111}$In delivered to each cell is sufficient to explain the toxicity observed, assuming that decays occur in the cytoplasm or the cell surface. When it is considered that the effect of chronic exposure to cell-bound radioactivity is expected to be approximately 3-fold less effective than brief irradiation by $^{137}$Cs (28) and that other approximations are required to calculate the dose from radiolabeled Abs, the results must be regarded as consistent with the effects of external beam irradiation.

Earlier experiments with radiolabeled Abs produced only weak toxicity (14, 29, 30), as we have summarized previously (7), but this was probably because the conjugates tested did not possess all of the properties required to achieve high levels of cell kill: high specific activity, use of residualizing radiolabels, and reactivity with high density antigens. Previous work on cell killing by LEE emitters used only $^{125}$I (12, 14). Whereas the published cellular S values (5) are slightly higher for $^{125}$I than for $^{111}$In, the difference is small, and the long half-life of $^{125}$I means that, for the same molar conjugation ratio (radionuclide to Ab), the specific activity will be much greater for $^{111}$In than for $^{125}$I. The earlier studies also emphasized the need for Ab internalization. However, our results with anti-EGP-1 strongly suggest that there is no need to invoke an unusual processing pathway for anti-EGFr because both Abs were similarly potent. In addition, our previous comparison of a rapidly internalized Ab with a slowly internalized Ab indicated that there was no detectable advantage of Ab internalization (9). The reason for this was discussed previously in detail, but a key consideration is probably the fact that the nucleus is typically located not in the center of the cell, but close to the cell membrane. Because $^{125}$I in the nucleus is approximately 100-fold more potent at cell kill than $^{125}$I in the cytoplasm (31, 32), the possibility of radionuclide delivery to the nucleus should be further discussed, especially because it was reported that both epidermal growth factor and antibodies to EGFr are delivered to the nucleus after binding to the cell surface (33, 34). Such nuclear transport is controversial because there is no well-established route by which an Ab bound to the cell surface can reach the nucleus. Although it is difficult to exclude the possibility that the toxicity observed in our experiments is due to a small fraction of the radionuclide that reaches the nucleus, there is no need to assume nuclear uptake because the level of toxicity observed can be explained by the calculated dose delivered from decays in the cytoplasm.

There is a significant technical difference in the dosimetry calculations between the nonadherent lymphoma cells and adherent carcinoma cells that should be pointed out. In experiments with lymphoma cells, dead cells (and their bound cpm) are pelleted together with the viable cells, and therefore the cpm determined at later time points (when cells began to die) are due to both viable and dead cells. Therefore, direct determination of cpm per viable cell could not be obtained, and the cpm per initial cell number was used for the dosimetry calculations. This was reasonable because there was little cell division in the wells treated with toxic levels of radiolabeled Abs. With the adherent cells, this problem had a much smaller impact because most dead cells evidently detached from the wells and were washed away. Therefore, we could calculate the cpm per viable cell.

Behr et al. (36, 37) also compared LEE emitters and $\beta$-particle emitters, using an Ab to colon carcinoma, anti-EGP-2 (which is closely related to EGP-1; ref. 35), to treat established subcutaneous xenografts of colon carcinoma. They performed a comparison of $^{125}$I versus $^{131}$I and of $^{111}$In versus $^{90}$Y. In both cases, at the maximum tolerated dose, the LEE emitter was a more effective therapeutic agent than the $\beta$-particle emitter. Thus, it seems possible that LEE emitters may be generally more effective than $\beta$-particle emitters at treating both large and small tumor burdens, but much further work is required to
investigate this possibility. The work of Behr et al. (36, 37) also raises the issue of the relative linear energy transfer (LET) of LEE emitters because the authors refer to both $^{125}\text{I}$ and $^{111}\text{In}$ as high LET radionuclides. It is well established that $^{125}\text{I}$ incorporated into DNA as iododeoxyuridine has a high LET effect, due to the >20 electrons emitted per decay (31). However, $^{125}\text{I}$ decays in the cytoplasm or the cell surface would not be expected to have a high LET effect because only a few electrons will reach the nucleus, and those that do will be scattered over a large volume. Indeed, studies with $^{125}\text{I}, ^{111}\text{In}, ^{67}\text{Ga}$, and other radionuclides have directly demonstrated that LEEs emitted in the cytoplasm have a low LET effect (32, 38–40). Also, our comparison of LEE emitters and β-particle emitters for single cell kill in vitro has not provided any indication of a high LET effect of the LEE emitters, which would have been demonstrated (8, 9).

These results, together with those published previously on lymphoma cell lines, strongly suggest that Ab conjugates with LEE emitters should be further evaluated as therapeutic agents for cancer. To better establish the therapeutic potential of this approach, more animal experiments are required. Inasmuch as this strategy is effective only with high-density antigen, some discussion of EGFR levels on tumors is appropriate. Whereas it is frequently stated that A-431 cells express extremely high levels of EGFR, this is in comparison with normal human fibroblasts (41, 42). There are a considerable number of tumor cell lines that express EGFR at a level similar to that of A-431 cells (11, 42). Although it is difficult to obtain quantitative data on receptor expression on primary human tumors, it is clear that high, homogeneous expression is not uncommon, at least in certain tumor types (11, 43, 44). Therefore, the A-431 cell line may be a meaningful experimental model. Moreover, based on our results with anti-EGP-1, the results presented are probably applicable to most other Abs reacting with high-density antigens on human tumor cells.

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REFERENCES


In vitro Toxicity of A-431 Carcinoma Cells with Antibodies to Epidermal Growth Factor Receptor and Epithelial Glycoprotein-1 Conjugated to Radionuclides Emitting Low-Energy Electrons

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