In vitro cytotoxicity of carcinoma cells with $^{111}$In-labeled antibodies to HER-2

Rosana B. Michel, Philip M. Andrews, Mary Ellen Castillo, and M. Jules Mattes

Center for Molecular Medicine and Immunology, Belleville, New Jersey

Abstract

Antibodies conjugated to radionuclides emitting low-energy electrons, which include Auger electrons and some conversion electrons, were recently shown to efficiently kill cells bearing a high density of the antigen recognized. The primary purpose of this study was to determine if such killing could be obtained with anti–HER-2 antibodies conjugated to $^{111}$In, using the chelator benzyl-diethylenetriaminepentaacetic acid, or $^{125}$I. Target cells were the breast carcinoma SK-BR-3 and the ovarian carcinoma SK-OV-3.ip1. In preliminary experiments, antibody accumulation and catabolism during a 2- to 3-day incubation with antibody was investigated. The level of antibody uptake, in terms of molecules per cell, was high enough such that killing seemed feasible. With an $^{125}$I label, but not an $^{111}$In label, increasing the antibody concentration past a certain point caused a decrease in total antibody accumulation, which might be attributed to effects of antibody binding. To test for cytotoxicity, cells were incubated for 2 days with the labeled antibody, then assayed for colony-forming units conditions in which the particular epitope of the HER-2 antigen that is recognized. The primary purpose of this study was to determine if such killing could be obtained with anti–HER-2 antibodies conjugated to $^{111}$In, using the chelator benzyl-diethylenetriaminepentaacetic acid, or $^{125}$I. Target cells were the breast carcinoma SK-BR-3 and the ovarian carcinoma SK-OV-3.ip1. In preliminary experiments, antibody accumulation and catabolism during a 2- to 3-day incubation with antibody was investigated. The level of antibody uptake, in terms of molecules per cell, was high enough such that killing seemed feasible. With an $^{125}$I label, but not an $^{111}$In label, increasing the antibody concentration past a certain point caused a decrease in total antibody accumulation, which might be attributed to effects of antibody binding. To test for cytotoxicity, cells were incubated for 2 days with the labeled antibody, then assayed for colony-forming units with a limiting dilution assay. SK-BR-3 cells were strongly killed (3 logs) by antibody 21.1, and 100% kill was obtained by combining two noncompeting antibodies to HER-2 (21.1 and 4D5). SK-OV-3.ip1 cells were more resistant to killing, but use of the two-antibody mixture produced a surviving fraction of 0.002. $^{111}$In-labeled antibodies to other high-density antigens, epithelial glycoprotein-1 and epithelial glycoprotein-2, also killed these target cells. In contrast, unlabeled antibodies or a nonreactive-labeled antibody produced much less cytotoxicity. The same experiment with an $^{131}$I label (a $\beta$-particle emitter) resulted in much greater levels of nonspecific cytotoxicity and essentially no specific cytotoxicity. This approach may be effective for therapy of micrometastases. [Mol Cancer Ther 2005;4(6):927–37]

Introduction

We recently showed strong, specific cytotoxicity of single tumor cells in vitro with radiolabeled antibodies, using antibodies conjugated with emitters of low-energy electrons (1–4). These low-energy electrons include both Auger and conversion electrons, and have energies in the range of 10 to 40 keV, which is optimal for specific single-cell kill (5). The radionuclides that were used are primarily $^{111}$In, $^{67}$Ga, and $^{125}$I. High antigen densities are required because a large number of decays is necessary for strong cytotoxicity. This approach was also effective in in vivo mouse xenograft models of B-cell lymphoma, because antibodies to CD74 or CD20, conjugated with $^{111}$In or $^{67}$Ga, were effective therapeutic agents under conditions in which the $\beta$-particle emitters $^{90}$Y, $^{177}$Lu, or $^{131}$I were much less effective (6–8). The key difference between low-energy electron emitters and $\beta$-particle emitters is the much greater nonspecific toxicity of the latter, which means that the maximum dose that can be injected into a mouse is ~10-fold lower. Following our initial results with B-cell lymphoma, using antibodies to CD74, CD20, and HLA-DR, experiments were done with the vulvar carcinoma cell line A431, using antibodies to epidermal growth factor receptor and epithelial glycoprotein-1 (EGP-1; ref. 9). Strong, specific killing was shown with $^{111}$In-labeled antibodies (4). Whereas $^{131}$I antibodies could also efficiently kill the cells, a large part of this killing was due to nonspecific irradiation of the cells due to decays occurring in the medium. These data showed that low-energy-electron emitters could be effective on adherent target cells, which is significant because the shape of these cells would be expected to make them considerably more difficult to kill than the spherical lymphoma cells. It was important to show that similar results could be obtained with other antibodies (to different antigens) and on other target cell lines; in the current study, we have extended this work to antibodies to HER-2. The HER-2 antigen is often overexpressed on carcinomas of the breast (10) and other histologic types (11), and some unconjugated antibodies to HER-2 inhibit cell growth (11, 12). The physiologic effect of an antibody strongly depends on the particular epitope of the HER-2 antigen that is recognized. The humanized antibody trastuzumab, derived from the mouse antibody 4D5, was shown to be therapeutically effective on human breast carcinomas (13, 14). Because the antigen is frequently expressed at
high densities, it seemed possible that it might be a suitable target for antibodies conjugated to low-energy-electron emitters, and we show herein that this is the case.

In as much as we use antibodies to two different epitopes of HER-2, the epitope structure of the antigen should be considered. The antigen has an extracellular region of ~650 amino acids (11). In a study of 11 antibodies to the antigen, Xu et al. (15) identified a complex epitope pattern in that the epitopes were overlapping and grouped in a linear array. This result, which seems somewhat unusual, is probably simply a result of the fact that so many antibodies were tested. Four distinct epitopes were defined (15). Of the two antibodies used herein, 4D5 was shown to recognize an epitope containing amino acids 529 to 627 (12), close to the terminus of the molecule (amino acids 1–241). These two antibodies, therefore, react with the same, or closely related, epitopes.

Materials and Methods

Cell Lines, Antibodies, and Radiolabeling

The SK-BR-3 cell line, a breast carcinoma, was obtained from the American Type Culture Collection (Rockville, MD). The ovarian carcinoma SK-OV-3.ip1, provided by Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX), is a subline of SK-OV-3 (an American Type Culture Collection line) that was selected for i.p. growth in nude mice (18). It was reported to have ~2-fold higher expression of HER-2 than the original SK-OV-3 cell line. Cells were grown as previously described (19). They were tested routinely for Mycoplasma contamination using the Mycotect Assay (Life Technologies, Grand Island, NY) and were negative. Hybridomas 21.1 (also called e21; ref. 16) and 4D5, producing antibodies reactive with HER-2, were obtained from American Type Culture Collection (HB-11601 and CRL-10463, respectively). Both are IgG1s. The 4D5 hybridoma, as obtained from American Type Culture Collection, was found to contain >90% of cells that produced no antibody, as detected by an ELISA assay for mouse IgG1. Therefore, it was subcloned twice to obtain a subclone that consistently produced antibody. Hybridoma RS7, producing an IgG1 antibody to EGP-1, and RS11, producing an IgG1 antibody to EGP-2, were previously described (9, 20). The nonreactive control antibody used was either MN-14, an IgG1 reactive with carcinoembryonic antigen, provided by Immunomedics, Inc. (Morris Plains, NJ), or the myeloma protein MOPC-21, from an American Type Culture Collection hybridoma. Hybridomas were grown in SFM tissue culture medium (Life Technologies) containing 0.5% low-IgG fetal bovine serum (Hyclone, Logan, UT), and antibody purified from 3 to 4 L of spent medium by chromatography on Protein A-Sepharose (Amersham Pharmacia, Piscataway, NJ) by standard methods. Antibodies were labeled with $^{125}$I and $^{131}$I by the chloramine-T method, and with $^{111}$In with the chelator isothiocyanate-benzyl-diethylenetriaminepentaacetic acid, as described previously in detail (21). For $^{111}$In labeling, 100 µg antibody was routinely labeled with 10 to 12 mCi $^{111}$In. Use of this high amount of $^{111}$In caused the labeling efficiency to decrease, but was used to maximize the specific activities obtained, and essentially all of the remaining free $^{111}$In was removed by passage over a PD10 gel filtration column and run with tissue culture medium, which was used also for the purpose of buffer exchange. To label mixtures of two to three antibodies, equal amounts of the antibodies were mixed and 100 µg of the mixture were labeled. Labeled preparations were analyzed by instant thin-layer chromatography or by gel filtration high-performance liquid chromatography, or both, as previously described (22), to determine the level of radioactivity not bound to the antibody, which was always <10% and usually <5%. The specific activity was generally 40 to 70 mCi/mg for $^{111}$In and 10 to 20 mCi/mg for $^{131}$I. Representative preparations of radiolabeled antibodies, with each radiolabel, were tested for immunoreactive fraction (percentage bindable) by incubating with a large excess of cells. Control tubes had excess unlabeled antibody added to block specific binding and, therefore, to indicate the level of nonspecific binding: Specific binding was calculated by subtraction. In these experiments, $^{125}$I was used instead of $^{131}$I for convenience, because in previous experiments we have observed no difference in immunoreactivity between these two iodine isotopes at the specific activities used. The specific binding was as follows (for $^{111}$In and $^{125}$I, respectively): 21.1, 76.9% and 52.8%; 4D5, 68.1% and 70.9%; anti–EGP-1, 54.1% and 60.7%; and anti–EGP-2, 68.6% and 62.0%.

Antibody Processing Experiments

These methods were described previously in detail (19). Briefly, cells were plated 1 day before use in a 96-well plate. Radiolabeled antibody was added and cells were incubated for 2 hours at 37°C. Control wells in every experiment contained a large excess of unlabeled antibody to show the specificity of antibody binding, which was at least 90% specific. After washing the wells, tissue culture medium was added and incubation continued for 2 to 3 days. At various time points, as indicated, 100 µL of supernatant was collected, counted for radioactivity, and then precipitated by cold 10% trichloroacetic acid to distinguish between intact (trichloroacetic acid precipitable) and degraded (trichloroacetic acid soluble) counts per minute (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 assayed for cell-bound cpm.

Antibody Uptake at Saturation in a Prolonged Antibody Incubation

These assays also were previously described in detail (23). Briefly, cells were incubated continuously with near-saturating concentrations of radiolabeled antibody. The antibody concentrations required were selected in
preliminary experiments. Experiments routinely included two concentrations to show that a 2-fold increase in the antibody concentration did not significantly increase the amount of antibody bound. At various time points, for 2 to 3 days, cell-bound cpm was determined. From the specific activity of the antibody used, and correcting for the percentage of non–protein-bound radioactivity (always <10%), the number of antibody molecules bound was calculated. From cell counts, determined from other wells, the number of antibody molecules bound per cell was calculated.

**Cytotoxicity Assays**

Cells (2 x 10^4) were plated in 96-well plates in 0.15 mL and allowed to adhere 1 day before application of the antibodies. After aspirating the medium, serial dilutions of antibody were added to quadruplicate wells, also in 0.15 mL, and plates were incubated for 2 days at 37°C. Control wells had no antibody added. At day 2, one well of each set was used for cloning. The well was washed twice with 0.25 mL tissue culture medium and then once with PBS. After adding 0.1 mL of trypsin, plates were incubated for 10 minutes at 37°C. The cells were all rounded at this time and readily collected as a single-cell suspension by repipetting. Because SK-BR-3 cells (but not SK-OV-3ip1 cells) had a tendency to aggregate after trypsinization, the trypsin used had a higher EDTA concentration than the standard trypsin, 3.0 mmol/L compared with 0.53 mmol/L. This reduced, but did not completely eliminate, the aggregation problem, but the aggregates were small and rare enough such that the results would not be substantially affected. Trypsinized cells were transferred to a tube containing 2 mL of medium, pelleted, and resuspended in medium. Two colony-forming unit (CFU) assays were used. In earlier experiments, serial dilutions of the cell suspension were plated in 60-mm-diameter Petri dishes, using an overlay of agar, as described previously in detail (4). In later experiments, serial dilutions were plated in 96-well plates as follows. Cells were suspended in 20 mL, and five serial dilutions were made by transferring 5 mL into 15 mL. The medium used had higher than usual concentrations of fetal bovine serum: 12.5% for SK-OV-3 and 20% for SK-BR-3. Each dilution (0.2 mL) was plated in 48 wells of a 96-well plate, and cultured for 19 to 21 days. With this long incubation time, there was substantial evaporation in the outer wells (despite the use of a humid incubator), so only the inner wells were scored, 30 wells for each dilution. Wells were scored as either positive or negative for growth of a colony of ≥50 cells. (In our experience, this is easier than counting colonies in a Petri dish, which is the reason that this assay was used.) Fixation and staining with methylene blue was as previously described (4), except that this was done by immersing the plates in trays filled with the reagents. The cell concentration was calculated using the Quality program. This program gives the CFU concentration per 0.2 mL (the volume plated per well) and SE, using the chi-squared method of Taswell (24). Although it has been argued that the maximum likelihood method is optimal for analysis of limiting dilution assays (25), the difference between these two methods is small (25) and we are not aware of readily available software that utilizes the maximum likelihood method.

A cell count was obtained from a control well, at the time of cloning, to calculate the cloning efficiency, which was essentially the same for both CFU assays used. The mean ± SD of the cloning efficiency was 54.3 ± 6.3% for SK-OV-3.ip1 and 20.6 ± 6.3% for SK-BR-3. However, as noted, the SK-BR-3 cell preparations contained some small clusters, despite the use of a high EDTA concentration. It is probably more accurate to give the cloning efficiency based on the number of clusters, rather than the number of single cells, and both values were determined. Based on the number of clusters, the cloning efficiency for SK-BR-3 was 31.5 ± 10.8%. Bound radioactivity was determined from additional wells, in triplicate, by harvesting with cotton swabs. In many cases, with 111In-labeled antibodies, 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, then recounted. The cpm at time 0 was then calculated from the decay curve.

For comparison with the radiolabeled antibodies, cells were irradiated with a 137Cs 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 CFU under the same conditions used in the other experiments. Radiation doses were also calculated for nonspecific toxicity from 131I (i.e., from decays occurring in the medium). The highest concentration tested was 400 μCi/mL. (This high concentration was used with a nonreactive control antibody to show essentially 100% nonspecific cytotoxicity.) Assuming 100% absorption of the electron energy, the radiation dose would be 3,576 cGy, over the 2-day period of radiation exposure, using the method previously described (1). 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% (26). Also, cells at the edges of the wells will receive a lower dose than cells in the center by a factor of ~2. Therefore, the estimated dose at this concentration is 1,606 to 3,212 cGy, depending on the location of the cell in the well.

**Results**

**Processing of Antibodies Labeled with 125I after Binding to Carcinoma Cell Lines**

Figure 1 shows antibody processing results with 125I-labeled antibodies to HER-2 or other antigens, for comparison, after binding to SK-BR-3 breast carcinoma
cells. This figure shows the level of retention of the bound radiolabel and the rate of catabolism, which is indicated by the release of labeled catabolites into the medium. Conventional iodine labels, as used here, are nonresidua-
ing, meaning that catabolites rapidly leave the cell. Results with anti-CD147 provide a useful comparison because this antibody binds strongly to all human cell lines tested (>100 different cell lines of diverse histologic types) and is always catabolized as slowly as any other antibody binding to the cell surface (19, 27); therefore, this antibody probably indicates the basal rate of membrane turnover. An antibody to the transferrin receptor, 5E9, is also included, as an example of a rapidly internalizing antibody that is catabolized relatively rapidly. Antibodies to EGP-1 and EGP-2 were also tested; these antigens are epithelial differentiation antigens that have been used for tumor targeting in patients and in experimental models (9, 20). As shown, anti–HER-2 was catabolized relatively rapidly, although slightly less rapidly than 5E9. The substantial difference between 21.1 and 5E9 in cell-bound cpm, evident in Fig. 1A, was partly due to the fact that dissociation of intact 5E9 was somewhat greater, as shown in Fig. 1C, but the major factor was the difference in the rate of catabolism. That is, the difference in the level of dissociation was only 6.4% of the initially bound cpm at 3 hours and 10.7% at 21 to 23 hours. Antibodies to both EGP-1 and EGP-2 were catabolized as slowly as anti-CD147. Anti-EGP-1 was catabolized more rapidly by other cell lines (28), but this property sometimes depends on the particular cell line. Similar experiments were also done with a second antibody to HER-2, 4D5: This gave results essentially the same as the results obtained with 21.1. We conclude that, to kill cells with radiolabeled anti–HER-2, there would be a considerable advantage of using residualizing radiolabels, such as most radiometals, which are trapped in lysosomes after catabolism of the antibody to which they were conjugated. However, it might still be useful to test radioiodine in cytotoxicity experiments, considering the convenience of this label. With an $^{125}$I label on 21.1, cell retention was 44.5% at 23 hours and 21.2% at 69 hours, which means that the radiolabel is retained sufficiently to deliver substantial amounts of radiation to the cells. Similar experiments were done with the SK-OV-3.ip1 ovarian carcinoma cell line, using antibodies to HER-2, CD147, EGP-2, and monomorphic HLA-ABC (antibody W6/32), and results were generally similar (data not shown). Catabolism of 21.1 by SK-OV-3.ip1 cells was faster than that of some of the other antibodies, but not exceptionally fast. In a typical experiment, the cell retention of $^{125}$I-21.1 was 41.9% at 21.5 hours and 27.8% at 45.5 hours.

**Antibody Uptake during a Prolonged Incubation with Saturating Concentrations of Radiolabeled Antibodies**

The key factor that determines the level of cytotoxicity with radiolabeled antibodies is the number of decays per cell. This is a function of the time course of antibody binding and retention per cell, which can be determined by incubating cells continuously, for up to 2 days, with a saturating concentration of radiolabeled antibody. In these experiments, only a trace label was used, so the amount of radioactivity was not high enough to kill the cells. The bound radioactivity is expressed in terms of “antibody molecule equivalents” because much of the bound radioactivity from the residualizing labels is in the form of catabolites trapped in lysosomes. Figure 2 shows results with two antibodies binding to SK-OV-3.ip1 cells. Antibodies were tested at saturating concentrations, which was determined in preliminary experiments. Unexpectedly, as shown in Fig. 2A, binding of $^{125}$I-labeled 21.1 decreased as the antibody concentration increased. This was observed consistently in five experiments. Peak binding occurred at 0.25 μg/mL, and binding was considerably lower at 1.0 μg/mL. This difference did not occur at the earliest time point (1 hour), and in fact at 1 hour binding was higher with the higher antibody concentration; however, by 4 hours, this difference was seen, and it remained evident for at least 24 hours. These differences were substantial: At 4 hours, the amount of antibody binding at 1.0 μg/mL was 2.3-fold lower than the amount binding at 0.25 μg/mL, and the difference at 22 hours was 2.9-fold. Use of an antibody concentration...
lower than 0.25 μg/mL (0.125 μg/mL) resulted in lower uptake (data not shown); thus, of the concentrations tested, peak uptake occurred at 0.25 μg/mL. It should be noted that, at a high antibody concentration of 1.0 μg/mL, antibody uptake decreased from 1 to 4 hours, whereas at the lower antibody concentration of 0.25 μg/mL, uptake increased over this time period. Another complexity of the binding curve, also shown in Fig. 2A, is that, at the higher antibody concentrations, after the decrease in antibody binding per cell from 1 to 24 hours, there was a subsequent increase (which might be called a rebound) from 24 to 48 hours.

These complex effects of varying antibody concentration were not seen with an 111In label: Binding was much higher with an 111In label than with the 125I label at any concentration, and decreasing the concentration of the 111In antibody resulted in the expected decrease in antibody molecules bound. At 4 hours, the antibody-equivalent uptake with the 111In label was 3.0-fold higher than the highest binding seen with the 125I label (at 0.25 μg/mL), and this difference further increased at later time points. A possible explanation for this complex binding behavior is presented below. These effects (the large difference between 111In and 125I, the decreased binding as the 125I-labeled antibody concentration was increased, and the decrease in 125I-labeled antibody binding from 1 to 24 hours) did not occur with the other antibodies tested, as exemplified by results with anti–EGP-2 (Fig. 2B). The effects also did not occur with the same antibody, 125I-21.1, binding to a different cell line, SK-BR-3 (data not shown). In this case, there was no decrease in bound antibody equivalents per cell from 1 to 4 hours or from 1 to 24 hours, at a concentration of 1.5 or 3 μg/mL, and binding reached a plateau of ~9 × 10^5 antibody equivalents bound per cell at 48 hours. These results indicate a significant difference between the two cell lines tested; the explanation for this difference is not known, but is discussed below. In conclusion, these data show that high levels of antibodies to both HER-2 and EGP-2 bind to SK-OV-3ip1 cells in a prolonged incubation, with a saturating concentration of antibody, which was 1 μg/mL for anti–HER-2 and 10 ng/mL for EGP-2. For 125I-labeled anti–HER-2, only, results with varying antibody concentrations are shown, because some of the antibody is catabolized, with only the radiolabel remaining within the cells. Antibodies were labeled with either 125I (open symbols) or 111In (filled symbols). Points, means of triplicates; bars, SD (but nearly all of the SDs are too small to be seen above the symbols). Results are representative of two experiments done with each radiolabeled antibody.

Cytotoxicity with 111In-Labeled Antibodies

Figure 3A shows cytotoxicity of SK-BR-3 with 111In-labeled antibodies. As shown, antibody 21.1 produced significant cytotoxicity, but the cytotoxicity curve began to develop a plateau at concentrations above ~20 μCi/mL, and 100% kill could not be obtained even at much higher concentrations. A curve of this shape can be attributed to antigen saturation, because the amount of antibody binding does not increase as the antibody concentration is increased. In fact, this is shown directly in Fig. 3B, which shows the bound cpm/well of the 96-well plate, for wells identical to those used for the clonogenic assay: Increasing the concentration of 111In-21.1 above 19 μCi/mL resulted in very little increase in the bound cpm. This result is also consistent with the binding experiments done previously, because the antibody concentration at 19 μCi/mL was 0.46 μg/mL, which was known to be near saturating. However, another possible explanation for a plateau in the cytotoxicity curve is that there is a fraction of cells resistant to killing by radiolabeled antibodies. To investigate this possibility, cytotoxicity experiments were done with a mixture of three antibodies to different antigens, each conjugated to 111In. The objective in this experiment was simply to bind as much 111In as possible to the cell surface. The antibodies combined were 21.1, anti–EGP-1, and anti–EGP-2. In preliminary experiments, some of which are shown in Fig. 1, antibodies to both EGP-1 and EGP-2 were shown to bind to high-density antigens on SK-BR-3 cells. Figure 3B shows that use of this antibody mixture was able to increase the bound cpm/well. Although the increase was only ~2-fold, this markedly enhanced the cytotoxicity, as shown in Fig. 3A, so that 100% killing was achieved. These results show that if enough 111In-labeled antibody is bound to the cell surface, 100% kill is possible. Results with a nonreactive antibody labeled in the same way are also shown in this figure: This produced very low levels of cytotoxicity, indicating that the killing was antigen-specific. There was some nonspecific cytotoxicity at very high

Figure 2. Uptake of antibodies to HER-2 (antibody 21.1; A) and EGP-2 (B) by SK-OV-3ip1 cells in a prolonged incubation, with a saturating concentration of antibody, which was 1 μg/mL for anti–HER-2 and 10 ng/mL for EGP-2. For 125I-labeled anti–HER-2, only, results with varying antibody concentrations are shown, because some of the antibody is catabolized, with only the radiolabel remaining within the cells. Antibodies were labeled with either 125I (open symbols) or 111In (filled symbols). Points, means of triplicates; bars, SD (but nearly all of the SDs are too small to be seen above the symbols). Results are representative of two experiments done with each radiolabeled antibody.
antibody concentrations: At 1.85 mCi/mL, which is outside the scale of the figure, the surviving fraction was 0.00418. But the specific antibodies were at least 40-fold more potent than the nonreactive antibody. Control experiments with unlabeled antibodies are described below.

To increase the cpm delivered by antibodies to HER-2, we combined two antibodies to different epitopes of the antigen, namely 21.1 and 4D5. By combining these two 111In-labeled antibodies, the level of cpm/well and the level of cytotoxicity were both increased substantially, and 100% kill was obtained (Fig. 3A and B). However, there was no perfect correlation between bound cpm and cytotoxicity. Thus, when the concentration of mixed 4D5 + 21.1 was increased from 56 to 167 $\mu$Ci/mL, there was no increase in the bound cpm, yet the cytotoxicity markedly increased. This pattern has been seen frequently in similar experiments; the explanation is not known, but is discussed below.

Similar experiments were done with SK-OV-3ip1 cells, primarily because this cell line grows better in immunodeficient mice (nude or SCID) than SK-BR-3, and in vivo therapy studies are planned. As shown in Fig. 4A and B, reactive antibodies produced specific cytotoxicity, but the absolute level of killing was lower than that obtained with SK-BR-3 cells and 100% kill was not obtained. The upward bend in the curves, with all of the antibodies tested, is, again, a consequence of the fact that antigen saturation was reached, as confirmed by the data shown in Fig. 4C. Figure 4B shows the same data as shown in Fig. 4A, but with a different scale, to include results with higher antibody concentrations. At these higher concentrations, the surviving fraction decreased to $\sim 1 \times 10^{-3}$, with the mix of 4D5 + 21.1, but there was also considerable

\[ \text{Fraction surviving} \]
nonspecific cytotoxicity at these high concentrations. A comparison between the reactive antibodies and the nonreactive antibody shows considerable specificity in the cytotoxicity observed: For a cell kill of 95%, the mix of 4D5 + 21.1 was 12.9-fold more toxic than the nonreactive antibody. As with SK-BR-3 cells, the level of killing was markedly enhanced by combining multiple antibodies to different antigens or to different epitopes on the HER-2 antigen. The level of bound cpm/well in these experiments is shown in Fig. 4C. Binding of anti-HER-2 antibodies (as indicated by bound cpm/well or bound cpm/cell) was only slightly lower than that obtained with SK-BR-3 cells (shown in Fig. 3B), in contrast to the much lower levels of cytotoxicity.

Table 1 is a summary of the level of cpm uptake obtained with 111In-labeled antibodies on the two cell lines used. The purpose of this table is 2-fold: (a) to provide the estimated cpm/well, which is the most useful parameter for comparison with other studies using different antibodies and cell lines, and (b) to facilitate comparison between SK-BR-3 and SK-OV-3.ip1. The values shown were obtained from the same cytotoxicity experiments described above, from three replicate wells, and were obtained at the time of cloning, which is after 2 days of antibody incubation. From similar experiments done with nonreactive antibodies, there was very little nonspecific binding, as shown in Figs. 3 and 4, indicating that >99% of the antibody binding was antigen-specific. The left side of Table 1 provides the bound cpm/well of the 96-well plate, and the right side gives an estimate of the bound cpm/cell. Although serial 3-fold dilutions of each antibody were tested, only the peak uptake is shown; this was not always obtained at the highest antibody concentration (although binding at the highest antibody concentration was always quite close to the peak). A possible reason for this effect is presented in Discussion. Much of the variation between replicate experiments, shown by the ranges given in Table 1, can be attributed to variation in the specific activities of the antibodies used. For example, one label of the mix of three antibodies (21.1 + anti–EGP-1 + anti–EGP-2) was prepared at the highest possible specific activity, by using 20 mCi to label 100 µg of antibody (twice as much as usually used). This resulted in a specific activity of 97 mCi/mg, which is unusually high, and is responsible for the very high cpm uptake with this antibody mixture in one of the experiments with each cell line. The calculation of cpm/cell uses only a mean cell number at the time of adding the antibody, derived from similar experiments, and is intended only to provide a rough estimate. The mean cell number was 2.10 × 10^4 for SK-BR-3 and 2.17 × 10^4 for SK-OV-3.ip1. By comparing the cpm uptake of SK-OV-3.ip1 versus SK-BR-3, it is evident that binding to SK-BR-3 was slightly higher, but the difference is not enough to explain the difference in cytotoxicity. Thus, the mix of three antibodies (21.1 + anti–EGP-1 + anti–EGP-2) bound in large amounts to SK-OV-3.ip1 cells, but was still not able to produce 100% kill (although strong killing was obtained); the binding of fewer cpm to SK-BR-3 cells produced 100% kill. These data support the previous conclusion that SK-OV-3.ip1 cells are more resistant than SK-BR-3 cells to this type of cytotoxicity.

**Cytotoxicity with 131I-Labeled Antibodies**

Figure 5 shows results of similar experiments with 131I-antibodies on SK-OV-3ip1 cells. Although this is a nonresidualizing radiolabel, and cumulative uptake per cell will therefore be less, it seemed interesting to test this β-particle emitter for comparison. With anti–EGP-2 labeled with iodine, binding is at a high level, in terms of antibody equivalents bound per cell, after a prolonged antibody incubation (Fig. 2). Binding of iodinated anti–HER-2 is lower than with an 111In label, but is still substantial. As shown in Fig. 5A, 131I-labeled antibodies killed cells effectively, but the killing was essentially all nonspecific. As discussed previously (1, 4), this can be attributed to nonspecific irradiation from decays occurring in the medium. It should be noted that the 131I-labeled nonreactive antibody was ~6.6-fold more potent than the same antibody labeled with 111In, based on the mean concentration required to produce 99% kill of SK-OV-3.ip1 cells. Specific binding of the reactive antibodies tested was shown in Fig. 5B, but the level of specific cpm binding per well (and per cell) was much less than that obtained with 111In-labeled antibodies, due primarily to the lower

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<th>Estimated peak bound cpm/cell</th>
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<td>SK-OV-3.ip1</td>
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NOTE: The mean cpm/well of triplicate wells of a 96-well plate was determined after a 2-day incubation with radiolabeled antibody. Serial dilutions of the antibodies were tested, but values are shown only for the single concentration that produced the highest bound cpm, which was not always the highest concentration tested, as discussed in the text. Values shown are the ranges obtained in two to three experiments with each antibody and cell line. The peak cpm/well was calculated by dividing the bound cpm/well, shown on the left, by an estimate of the cell number per well, which is the mean number of cells at the time of adding antibodies, determined in similar experiments. The mix of three antibodies consisted of 21.1 + anti–EGP-1 + anti–EGP-2. Abbreviation: nd, not done.
specific activity of the 131I conjugates. As a consequence of these two factors, the radiation dose delivered from the medium was probably higher than the radiation dose delivered from specifically bound antibodies, and this can explain the results observed. This is true even for the mix of three antibodies (21.1 + anti–EGP-1 + anti–EGP-2), which was used to maximize the level of 131I binding to the cells.

Cytotoxicity with Unlabeled Antibodies

To show that the cytotoxicity observed was a result of radiation, control experiments were done with unlabeled antibodies, at 5 μg/mL, which is higher than the highest concentration of radiolabeled antibody that was used. Antibodies were tested alone and in combination, as done with radiolabeled antibodies. The unlabeled antibodies had at most a small cytotoxic effect, much less than the effect of radiolabeled antibodies. Thus, the mixture of 21.1 + 4D5 reduced the surviving fraction to 0.77 with SK-BR-3 cells and to 0.79 with SK-OV-3.ip1 cells. The mixture of three antibodies (21.1 + anti–EGP-1 + anti–EGP-2) was perhaps slightly more cytotoxic, and reduced the surviving fraction to 0.46 with SK-BR-3 cells and to 0.73 with SK-OV-3.ip1 cells. Such low levels of killing are barely detectable with the clonogenic assay used.

Discussion

The results presented further establish the general conclusion that antibodies conjugated to low-energy-electron emitters can efficiently and specifically kill single cells, which can be considered a model for the treatment of micrometastatic disease. These results with anti–HER-2 antibodies, combined with previous results with anti–epidermal growth factor receptor, anti-CD20, anti–HLA-DR, and anti-CD74, show that this approach can be effective with many antibodies of clinical interest, although it should be emphasized that high-density antigens are required. Our results also showed that a mixture of two antibodies was much more potent than a single antibody, which could be attributed to the increased cpm bound per cell. This advantage may be further increased by using...
more than two antibodies, because both HER-2 and epidermal growth factor receptor have large extracellular domains, and as many as five distinct epitopes have been characterized (15, 30). The steep dose-response curve, as shown in Fig. 4, means that a 2-fold or 3-fold increase in the cpm bound can result in a much larger increase in the level of killing.

Given the dependence of cytotoxicity on high levels of antibody binding, it is important to consider whether autologous tumors are likely to express levels of antigen comparable with those expressed on the cell lines used. Overexpression of HER-2 by carcinomas of the breast, ovary, and other organs has been extensively investigated and well established (10, 14), but the assays used to measure protein levels are semiquantitative and do not provide the number of sites per cell. Judging from the intensity of immunohistologic staining, HER-2 abundance is often high and usually homogeneous (14, 31). More quantitative assays have been used to measure gene amplification, which correlates strongly with protein expression (10, 14). The level of gene amplification in the SK-BR-3 cell line was determined to be 4- to 8-fold (32), which is at a level similar to that in many tumors having HER-2 amplification: Of such tumors, more than half have >5-fold amplification, and this constituted 14.3% of primary breast cancer specimens in the original study (33), which has been confirmed (10). It should also be noted that 4 of 11 cell lines tested by Kraus et al. (32) have levels of HER-2 expression comparable with the level on SK-BR-3 and SK-OV-3. Therefore, it seems that the cell lines used here express a level of HER-2 that would be found on autologous tumors having a high level of overexpression.

In as much as radioimmunotherapy studies have primarily utilized β-particle emitters, and both of the radiolabeled antibodies approved by the Food and Drug Administration for clinical use contain β-particle emitters (131I and 90Y), it is important to discuss the potential advantages of low-energy-electron emitters over β-particle emitters, which was shown previously in vitro (1, 3, 4) and in vivo (6–8) and is further supported by the data presented above. A disadvantage of β-particle emitters is their high level of nonspecific cytotoxicity: 131I had ~7-fold greater nonspecific cytotoxicity than 111In (calculated in terms of the μCi/mL required for 99% kill of SK-OV-3.ip1 cells from the data shown in Figs. 4 and 5), and, probably as a consequence, did not display any specific cytotoxicity. Whereas it can be argued that 131I does not provide a fair comparison, because it is a nonresidualizing label, similar results have been obtained with residualizing β-particle emitters, such as 90Y or 177Lu (1, 3, 4, 6, 8). Nonspecific cytotoxicity from 90Y (1) and 177Lu (8) is similar to that from 131I, and an increase in the retention of antibody catabolites, with anti–HER-2 antibodies, is not of sufficient magnitude to have a major impact.

Although SK-OV-3.ip1 cells were efficiently killed by 111In-labeled mixed 21.1 plus 4D5, the level of resistance of this cell line to radiolabeled antibodies was impressive. For example, with 111In conjugated to the mix of three antibodies (21.1 + anti–EGP-1 + anti–EGP-2), there were 2 × 10⁶ to 7 × 10⁹ cpm bound per well (the range in individual experiments), yet 100% killing was not obtained (the lowest surviving fraction was 9.2 × 10⁻³). Such high levels of bound cpm killed SK-BR-3 or A-431 cells much more effectively in this study and in our previous investigation (4). It is interesting to consider why SK-OV-3.ip1 cells were so resistant. One possibility is that these cells may be generally more resistant to radiation than most carcinoma cell lines. This has, in fact, been reported by others (34, 35), albeit with different sublines of the cells. But if this is the case, it should be evident from the response to 137Cs external beam irradiation and also from the response to nonspecific irradiation from unbound radioactivity in the medium (conjugated to a nonreactive antibody). In fact, there was little indication of such a difference in our experiments. A second possibility is that clustering of cells is a key factor that affects the dose delivered to the nuclei of target cells. Growing normally, adherent to plastic, SK-BR-3 cells have a strong tendency to form three-dimensional clusters, with rounded cells on top of and attached firmly to the spread-out cells beneath. This is much less the case for SK-OV-3.ip1 cells. For cells in clusters, much of the radiation dose to the nucleus, from low-energy-electron emitters, is from decays that occur on adjacent cells (called cross-dose). A similar situation was analyzed for three-dimensional tumor aggregates, and it was shown that even for relatively small clusters, the cross-dose from 111In may be more important than the self-dose (meaning decays originating from the same cell; ref. 36).

The results of our antibody processing experiments are consistent with previous results from other laboratories (37), although we are not aware of previous studies of long-term uptake of radiolabeled antibodies to HER-2. It is well established that some antibodies to HER-2 induce rapid internalization and catabolism of the antigen and the antibody (12, 37, 38). In our experiments, catabolism of anti–HER-2 antibodies occurred at a moderate rate, although less rapidly than with two other antibodies tested. The occurrence of catabolism means that uptake and accumulation will increase if a residualizing radiolabel is used, and this was shown directly in Fig. 2. However, uptake of 125I was still high, with a peak of 5.6 × 10⁵ antibody molecule equivalents bound per SK-OV-3.ip1 cell for antibody 21.1. In addition to catabolism, another key factor that will affect cumulative antibody uptake is the antigen synthetic rate, which may also be affected by antibody binding. If catabolized antigen is not rapidly replaced by newly synthesized antigen, then there will be down-regulation of cell surface expression; if it is rapidly replaced, then catabolism can potentially result in an increase in antibody uptake.

Figure 2 shows complex binding results with an 125I–anti–HER-2 antibody: cumulative uptake decreased as the antibody concentration increased, and, at a high antibody concentration, binding per cell at 24 hours was much lower than it was at 1 hour. Such effects can probably be
attributed to antibody-induced down-regulation of the level of antigen expression, which has been described with this antigen (12, 39). The decrease in antibody binding as the antibody concentration was increased can be attributed to the fact that antigen down-regulation requires a relatively high, near-saturating antibody concentration. These effects did not occur with an 111In label, probably because the mechanism of down-regulation is catabolism, and 111In catabolites are trapped inside the cell (40).

Previous studies with anti–HER-2 antibodies showed that the degree of down-regulation was relatively low even for those antibodies that are rapidly internalized, suggesting that the cells are able to partially replace the antigen that is internalized and destined for catabolism. For example, Harwerth et al. (38) incubated cells for 2 days with saturating concentrations of a mixture of two antibodies, which induced rapid antigen catabolism; however, the level of antigen expression on the cells was reduced by only 45%. Another possible explanation for the differences between 111In and 125I is that the particular labeling method affected the processing of the antibody, but there is no reason to suspect that this is the case, and we have not observed such differences with other antibodies labeled with 111In versus iodine (2, 3, 40). The difference between cell lines should also be noted: The unexpected binding effects seen with SK-OV-3.ip1 cells, as described above, were not seen with SK-BR-3 cells, suggesting that there is less down-regulation of the antigen on SK-BR-3 cells under the conditions used. Shawver et al. (39) previously examined antibody-induced HER-2 down-regulation on the same two cell lines (albeit with a different clone of SK-OV-3) and did not find differences between them. However, the assays used were different, because these authors examined labeled antigen as opposed to labeled antibody, and there were also differences in the antibody concentrations used, and in the particular antibodies used, which can affect the results of down-regulation experiments (39).

The cytotoxicity observed was primarily an effect of the radiation delivered, as shown by results obtained with unlabeled antibodies, which produced only a small reduction in the surviving fraction. Such results are consistent with previous reports of growth inhibition with unconjugated antibodies to HER-2, which showed a maximum growth inhibition of about 50% to 67% (39, 41). Xu et al. (15) reported cytotoxicity levels of up to about 80% with the most active of the anti–HER-2 antibodies tested, but they assayed anchorage-independent growth in soft agar, as opposed to growth adherent to plastic, and it is known that the toxic effects of unlabeled antibodies to HER-2 are more pronounced with the soft agar cloning assay (17, 41). Cytotoxicity results depend strongly on the particular antibody used (38). Antibody 454C11, which seems (on the basis of a limited comparison) to be most similar to our antibody 21.1, had virtually no toxic effect in the study of Xu et al., whereas 4D5 is among the more toxic anti–HER-2 antibodies (12, 41).

In the previous study with anti–epidermal growth factor receptor, we calculated the cGy dose per cell that was delivered by bound radiolabeled antibodies, from the actual cpm/cell at various time points (4). We did not perform such calculations in the current study, but the estimates provided in Table 1, compared with the cpm/cell obtained in the epidermal growth factor receptor study (4), show that the level of uptake observed is sufficient to deliver high radiation doses to the nucleus, and is consistent with the high levels of cell kill. One factor that tends to confound dose estimates is the fact, mentioned under Results, that higher antibody concentrations sometimes produced greater levels of killing with no increase in the cpm bound. A likely explanation is that some dead cells detach from the surface of the plate and are washed away, which would cause an underestimate of the true bound cpm/well.

Because of its preferential expression on tumor cells, HER-2 has been tested as a target for antibodies conjugated to a range of radionuclides, drugs, and toxins (16, 17, 42). Each of these conjugates has certain advantages and disadvantages. For example, the low level of HER-2 expression in normal hepatocytes caused serious hepatic toxicity in patients treated with a potent antibody-toxin conjugate (42). Because of the low level of antigen, and also because hepatocytes are relatively radiation-resistant cells, such toxicity would probably be less of a problem with antibodies conjugated to low-energy-electron emitters. Another advantage of radionuclides is that a large fraction of the activity will decay in the blood before uptake by normal cells involved in clearance of circulating antibody. For low-energy-electron emitters, but not for β-particle emitters, such decay in the blood will tend to limit bone marrow toxicity.

Further investigations are required to evaluate the potential of this strategy. Because high specific activities are required, it would be most suitable for antibodies that can efficiently reach tumor target cells when injected at a relatively low protein dose. This is the case for anti–HER-2, which showed acceptably slow blood clearance in patients (which allows time for the antibody to reach tumor cells) at a dose of 20 mg (43). This factor is primarily a function of the level of antigen expression on normal tissues. The maximum tolerated dose of radioactivity will be relatively high, probably in the range of 800 mCi, but such a dose is not impossible, considering that patients have been injected with as much as 500 mCi of 125I on an antibody (44), and 4 Ci of 111In conjugated to octreotide, in multiple doses (45, 46).

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References


In vitro cytotoxicity of carcinoma cells with $^{111}$In-labeled antibodies to HER-2

Rosana B. Michel, Philip M. Andrews, Mary Ellen Castillo, et al.